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<p><b>(54) Title:</b> METHOD AND APPARATUS FOR RAPID NUCLEIC ACID SEQUENCING</p> <p><b>(57) Abstract</b></p> <p>An automated nucleic acid sequencer is provided comprising an oligomer synthesizer, a membrane unit array, a detector and a central computer. The synthesizer synthesizes and labels multiple oligomers of [arbitrary] predicted sequence, which are transported to selected membranes contained within a membrane unit array, where they are hybridized to sequencing patterns bound to the membranes. A detector detects the hybridized sequencing patterns and sends descriptions of those patterns to the central computer, which analyzes those descriptions to construct a nucleic acid sequence, predicts a next set of oligomers for subsequent hybridizations, and selects corresponding membranes for hybridization with each predicted oligomer. Under computer control, synthesis of multiple oligomers, hybridization within multiple membranes, detection of the resulting patterns on multiple membranes, prediction of next oligomers, and selection of corresponding membranes, proceed simultaneously in accordance with the steps of a method of automated sequencing. Also provided is a method for automated nucleic acid sequencing.</p>			

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METHOD AND APPARATUS FOR RAPID NUCLEIC ACID SEQUENCING

TECHNICAL FIELD OF INVENTION

This invention relates to a method and apparatus for automating the steps of nucleic acid sequencing. More particularly, this invention relates to a computer controlled sequencer that performs an iterative process of synthesizing sets of predicted oligomers, hybridizing each synthesized oligomer to sequencing patterns immobilized on a selected membrane contained in an array of membrane units, detecting the hybridized patterns, analyzing those patterns to construct nucleic acid sequences, and predicting next sets of oligomers and selecting corresponding membranes for subsequent iterations of synthesis, hybridization, detection, analysis, prediction and selection.

BACKGROUND OF THE INVENTION

For more than a decade, scientists have utilized nucleic acid sequencing techniques to identify and study genes and their products. Traditional DNA sequencing techniques utilizing chemical cleavage (Maxam and Gilbert, Proc. Natl. Acad. Sci. USA, 74, pp. 560-564 (1977)) and chain termination (Sanger et al., Proc. Natl. Acad. Sci. USA, 74, pp. 5463-5467 (1977)) are well known to those skilled in the art. Although different in principle, both techniques generate separate populations of labelled

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oligonucleotides that begin from a fixed point and terminate randomly at a particular type of residue. Gel electrophoresis of such populations generates "ladders" from which the DNA sequence can be read.

5         The academic and commercial demand for nucleic acid sequence information has increased to the point that it is now desirable to begin sequencing not just isolated genes, but entire genomes of microorganisms, plants and animals. For instance, 10 sequence information from the human genome, provided by the Human Genome Project, is expected to enable vast progress in disease diagnosis, treatment and drug design. A variety of sequencing methods are currently being employed in genomic sequencing efforts as well as 15 in small-scale sequence determinations.

       "Genomic sequencing," which was first developed in 1984, involves direct chemical modification of unlabelled genomic DNA, combined with complete restriction enzyme digestion and size 20 separation on a denaturing gel. Numerous co-migrating sets of DNA sequence ladders are produced that, after transfer to a membrane, can be visualized independently by probing the membrane with a single-stranded labelled probe specific for one end of one restriction fragment 25 within the genome [G.M. Church and W. Gilbert, "Genomic Sequencing", Proc. Natl. Acad. Sci. USA, 81, pp. 1991-95 (1984)]. Because the DNA sequence is determined directly from genomic DNA, without the necessity of intervening cloning steps, this technique also permits 30 study of naturally occurring DNA modifications and DNA-protein interactions.

       Recently, two new techniques have been developed that allow faster and more efficient DNA sequencing. The first, "multiplex DNA sequencing," 35 involves formation of artificial genomes from mixtures

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of sonicated genomic fragments inserted into multiple vectors adjacent to various "tag" oligonucleotides. The DNA is chemically cleaved, electrophoresed, transferred to membranes, and then probed repeatedly 5 with oligonucleotides complementary to the "tag" sequences in the vectors [G.M. Church and S. Kieffer-Higgins, "Multiplex DNA Sequencing," Science, 240, pp. 185-88 (1988)].

The second technique, "multiplex walking," is 10 described in O. Ohara et al., "Direct Genomic Sequencing Of Bacterial DNA: The Pyruvate Kinase I Gene Of Escherichia Coli," Proc. Natl. Acad. Sci. USA, 86, pp. 6883-87 (1989). This technique utilizes the genomic sequencing technique described above. When a 15 labelled oligomer is hybridized to a membrane containing the chemical cleavage products of restriction digested DNA, sequence appears wherever the oligonucleotide lies near a restriction site. For each restriction digest, sequence may be readable in either 20 the 5' or the 3' direction, depending on the location of the oligonucleotide relative to the restriction site. Confirming sequence appears in the lanes derived from other restriction digests. Thus, each probing reveals the sequence of one strand of DNA extending in 25 one or both directions from the location of the oligonucleotide. Sequence can be read from the hybridization site of the probe along the length of the restriction fragment, until extraneous sequences that originate from the next restriction site obscure the 30 sequence ladder. Sequence from the complementary strand is generated on separate membranes by using complementary oligonucleotides.

After the sequence from the first hybridization is determined, new probes are 35 synthesized, with their sequences being derived from

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that portion of DNA that contains restriction sites and that is located near the 5' and 3' ends of the newly generated sequence. When the membranes are rehybridized with the new probes, additional sequence 5 ladders are revealed. Repeated cycles of oligomer probe synthesis and subsequent reprobing permit sequence walking along the genome. [See O. Ohara et al., *supra*.]

Despite the development of the DNA sequencing 10 techniques described above, the cost and time required to sequence the number of nucleic acids now being studied presents a great hindrance to current research efforts. There exists, therefore, a great demand for faster, more efficient, and less expensive ways to 15 sequence DNA.

#### SUMMARY OF THE INVENTION

The present invention solves the problems referred to above by providing a method and apparatus for automating nucleic acid sequencing and for 20 increasing the speed and efficiency of nucleic acid sequencing.

It is an object of this invention to provide an automated sequencer that will perform nucleic acid sequencing through an iterative process of synthesizing 25 multiple predicted oligomers and hybridizing at least two of those oligomers to sequencing patterns bound to respective selected membranes.

It is a further object of this invention to provide an automated sequencer comprising an oligomer 30 synthesizer capable of synthesizing multiple oligomers simultaneously.

It is a further object of this invention to provide an automated sequencer comprising a plurality of membrane units, each of which includes a membrane

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containing immobilized nucleic acid sequencing patterns, the membrane being bonded to a rigid backing plate to enable multiple iterations of hybridization, washing and stripping of the membrane, and to enable

5 automated movement of the membrane.

It is a further object of this invention to provide an automated sequencer comprising a membrane unit array that stores a plurality of membrane units that are individually selected for automated

10 hybridization with a predicted oligomer, each stored membrane unit being separately connected to an automated fluid transportation system for providing oligomers and fluids to hybridize, wash and strip the membrane contained therein.

15 It is a further object of this invention to provide an automated sequencer comprising a detector to detect the hybridized sequencing patterns on the membranes and to provide descriptions of such patterns to a computer.

20 It is a further object of this invention to provide an automated sequencer comprising a computer that controls and coordinates the iterative, multiple operations of: synthesis of predicted oligomers; hybridization of each oligomer to sequencing patterns

25 immobilized on selected membranes; detection of hybridized patterns on the membranes; interpretation of detected patterns to construct nucleic acid sequences; prediction and synthesis of next oligomers; and selection of corresponding membranes for subsequent

30 iterations of hybridization, detection, interpretation, prediction, synthesis, and selection.

It is a further object of this invention to provide an automated sequencer that will perform nucleic acid sequencing through an iterative process of

35 synthesizing multiple predicted oligomers, hybridizing

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at least two of those oligomers to sequencing patterns immobilized on selected membranes contained within a plurality of membrane units, detecting the hybridized sequencing patterns, interpreting the patterns to  
5 construct nucleic acid sequences, and predicting next oligomers for subsequent iterations of the process.

It is a further object of this invention to provide a method for automated sequencing of a nucleic acid using a multiplex walking technique comprising the  
10 steps of synthesizing oligomers of predicted sequence, hybridizing synthesized oligomers to sequencing patterns bound to selected membranes, detecting hybridized sequencing patterns, analyzing detected patterns to construct a nucleic acid sequence,  
15 predicting the sequence of and synthesizing next oligomers and selecting membranes for subsequent hybridization, detection and analysis.

In accordance with the invention, an automated sequencer and method for automated sequencing  
20 are provided. The automated sequencer comprises an oligomer synthesizer, a membrane unit array, a detector and a central computer.

The oligomer synthesizer simultaneously synthesizes multiple oligomers of any chosen sequence  
25 in accordance with instructions received from the central computer. The synthesized oligomers are then purified and labelled by the automated synthesizer.

The membrane unit array contains a plurality of reusable membrane units. The membrane units, which  
30 are constructed separately, and preferably in advance of oligomer synthesis, include a membrane containing immobilized nucleic acid sequencing patterns, a backing plate to which the membrane is bound, a sheet that is attached to the backing plate and covers the membrane  
35 to form an enclosure, and a series of inlet and outlet

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tubes for permitting fluids to be transported into and out of the enclosed space.

A fluid transportation system conducts each labelled oligomer to a selected membrane unit contained

- 5 within the membrane unit array for hybridization. The fluid transportation system also conducts a series of fluids into and out of the membrane units for hybridizing, washing and stripping the membranes contained therein. An opposing moveable plate is also
- 10 provided to compress the cover sheet to restrict the layer of fluid between the membrane and the cover sheet to a desired thickness. These steps can proceed independently and simultaneously in the various membrane units contained in the membrane unit array.

- 15 15 The detector detects the sequencing patterns on the membranes elucidated by hybridization of the labelled oligomers, and provides descriptions of those patterns to the central computer. A detection element may be inserted between the cover sheet and the

- 20 moveable plate to enable detection of the hybridized sequencing patterns, or the membrane unit may be extended out of the membrane unit array to be presented to a detector for detection of the hybridized patterns on the membrane. After detection, each detected
- 25 membrane is stripped of the hybridized oligomer to enable subsequent hybridization with a next oligomer.

The central computer analyzes the detected patterns to construct a nucleic acid sequence, predicts a next set of oligomers for extending and checking the

- 30 30 determined nucleic acid sequence, directs the synthesizer to synthesize the next set of oligomers, and determines which membrane each predicted oligomer will be directed to for hybridization. The central computer further serves to control the operation of the
- 35 35 components of the automated sequencer in a coordinated

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manner, reducing the time required to determine the nucleic acid sequence. Under computer control, synthesis of multiple oligomers, transportation of oligomers and fluids, hybridization within multiple 5 membrane units, and detection of the resulting patterns on multiple membranes and construction of nucleic acid sequence data proceed rapidly and efficiently in accordance with the steps of the method of automated sequencing described herein.

10 BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects and advantages of this invention will be apparent from consideration of the following detailed description, taken in conjunction with the accompanying drawings:

15 FIG. 1 is a schematic representation of the principle components of the sequencer;

FIG. 2 is a combined block-pictorial diagram of one embodiment of the synthesizer of the present invention;

20 FIG. 3 is a cross-sectional view of a portion of the synthesizer;

FIG. 4a is a top elevational view of the trough module;

25 FIG. 4b is a front elevational view of the trough module;

FIG. 4c is a rear elevational view of the trough module;

FIG. 4d is an end elevational view of the trough module;

30 FIG. 5 is an end view of a portion of the synthesizer;

FIG. 6 is a bottom perspective view of the trough module and associated structures;

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FIG. 7 is a diagram of the reagent reservoir and drive system;

FIG. 8 is a partially exploded side elevational view of a solenoid-piston-reagent-tip  
5 assembly;

FIG. 9 is a wiring diagram of the OM-915 controller terminal board;

FIG. 10 is a schematic diagram of the valve block;

10 FIG. 11 is a schematic diagram of the synthesizer enclosure wiring;

FIG. 12 is a flow chart for a computer program suitable for use with the synthesizer;

15 FIG. 13 is a simplified perspective diagram showing the placement of the solenoid array with respect to the trough module;

FIG. 14 is a perspective view of a membrane unit array with one membrane unit extending therefrom for presentation to a second embodiment of the  
20 detector;

FIG. 15 is a second perspective view of a membrane unit array with one membrane unit extending therefrom and engaged with a second embodiment of the detector;

25 FIG. 16 is a perspective view of the preferred embodiment of the membrane unit and opposing cover plate;

30 FIGS. 17a and 17b are sectional views of the membrane unit and opposing cover plate showing their released and compressed positions during a cycle of hybridizing, washing and stripping;

FIG. 18 is a schematic representation of one embodiment of the fluid transportation system;

35 FIGS. 19a and 19b are sectional views of the membrane unit and opposing cover plate showing their

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released and compressed positions in connection with a first embodiment of the detector.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides an automated nucleic acid sequencer that is able automatically to perform the steps of multiplex genomic walking, thus greatly enhancing the efficiency and speed of nucleic acid sequencing. According to this invention, nucleic acid sequences are determined in an automated, iterative process of synthesis of multiple oligomers, hybridization of said oligomers to sequencing patterns immobilized on multiple membranes contained within a membrane unit array, detection and analysis of hybridized sequencing patterns, construction of a portion of a nucleic acid sequence, and analysis of the newly constructed sequence to predict a next set of oligomers to be synthesized, hybridized, etc., in order to extend and check the sequence.

Information obtained from the nucleic acid sequence data generated by the apparatus and method of this invention is useful to prepare nucleic acid molecules, including DNA, cDNA and RNA molecules, which have at least a portion of their sequence based on the aforementioned nucleic acid sequence data. Such prepared nucleic acid molecules may be useful in a variety of other methods, for example: methods comprising the steps of cloning, isolation, or manipulation of particular genetic sequences in tissues or other analytes; methods of determining the presence or absence of particular genetic sequences; methods of determining the presence or absence of any molecule or other thing associated with the presence or absence of particular genetic sequences; methods of diagnosis and detection of pathological or nonpathological conditions

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which may be associated with the presence or absence of particular genetic sequences or the presence or absence of things associated with those sequences; methods comprising the step of introducing a particular genetic 5 sequence into a host cell or organism; and any other method which comprises a step requiring knowledge and possession of a particular genetic sequence.

As used herein, a "nucleic acid sequencing pattern" is any pattern of nucleic acid molecules bound 10 to a membrane and capable of being hybridized with a nucleic acid probe to reveal a nucleic acid sequence ladder.

As used herein, a "nucleic acid sequence ladder" is the pattern of nucleic acid molecules which 15 could be identified by an effective end label and which results from size separation of the products of a sequencing reaction, so that the order of elements in the pattern corresponds to the positions of the bases in a nucleic acid sequence.

20 As shown in FIG. 1 and described more fully below, the principal components of automated sequencer 1-1 of this invention are central computer 1-10, synthesizer 1-20, membrane unit array 1-30 and detector 1-40.\* Central computer 1-10 not only assembles and 25 analyzes sequence, but also controls and coordinates the operation of all of the other components of automated sequencer 1-1.

Central computer 1-10 constructs nucleic acid sequences by processing descriptions of hybridized 30 sequencing patterns received from detector 1-40. Such processing includes the steps of recording the descriptions of hybridized sequencing patterns,

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\* The first number (i.e., the number preceding the hyphen) refers to the figure number. The second number 35 refers to the particular element of that figure.

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interpreting each such description to construct a portion of a nucleic acid sequence that corresponds to the description, and combining portions of nucleic acid sequences to construct longer nucleic acid sequences.

5 Based on analyses of these constructed sequences, computer 1-10 then predicts a next set of oligomers to be synthesized by synthesizer 1-20, and controls the synthesis of those oligomers.

In conjunction with the prediction and

10 synthesis of oligomers, computer 1-10 also selects a particular membrane unit 14-50, contained in membrane unit array 1-30 for hybridization with each predicted and synthesized oligomer. It will be apparent that hybridization of that next set of oligomers to nucleic acid sequencing patterns immobilized on the selected membranes produces additional sequence descriptions that, in turn, are used to extend or to check the previously determined nucleic acid sequence.

The control signals that computer 1-10

20 generates are represented generally in FIG. 1 as being transmitted to synthesizer 1-20, membrane unit array 1-30, and detector 1-40 over lines 1-12, 1-13 and 1-14, respectively. Lines 1-12, 1-13 and 1-14 are also used to provide feedback information to computer 1-10

25 concerning the status of synthesizer 1-20, array 1-30 and detector 1-40. Lines 1-14 also transmits sequence information from detector 1-40 to computer 1-10.

Any or all of lines 1-12, 1-13 and 1-14 may in fact communicate with additional computers,

30 microprocessors or microcontrollers that are integrated with synthesizer 1-20, array 1-30 or detector 1-40 to control the particular operations performed by those devices. Computer 1-10, however, has ultimate control over the operation of each of those devices. The 35 central control provided by computer 1-10 enables these

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component devices to operate in a coordinated, overlapping manner, thereby performing the series of steps required to construct nucleic acid sequences rapidly, automatically and efficiently. Accordingly, 5 the control provided by computer 1-10 effects a reduction in the total time required to determine the sequence of a nucleic acid molecule.

Synthesizer 1-20 is capable of simultaneously synthesizing a plurality of oligomers of any chosen 10 sequence in response to and in accordance with instructions received from computer 1-10 over line 1-12. By simultaneously synthesizing multiple oligomers, synthesizer 1-20 substantially decreases the time that would otherwise be required to synthesize 15 individual oligomers serially, and decreases the expense and complexity required to network multiple synthesizers, each capable of synthesizing only one oligomer at a time. In addition to synthesizing oligomers from constituent nucleotides, synthesizer 20 1-20 purifies the synthesized oligomers, labels the purified oligomers appropriately with radioactive or other detectable labels, and provides each labelled oligomer to fluid transportation system 1-21 for transfer to its respective selected membrane for 25 hybridization.

As described above, synthesizer 1-20 is capable of the simultaneous synthesis of a large number of different oligomers. Synthesizer 1-20 comprises a multiplicity of surfaces upon which each of such 30 oligomers can be synthesized. For purposes of illustration, synthesizer 1-20 is described as having six surfaces, although it may incorporate many more surfaces, thus allowing the simultaneous synthesis of many more oligomers.

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Synthesizer 1-20 also includes a number of troughs that hold reagents. The reagent troughs are preferably formed within an inert material such as plastic. The directed movement of a surface in and out 5 of a series of reagent troughs allows synthesis of an oligomer of any desired nucleotide sequence.

Synthesizer 1-20 further comprises a system of valves (e.g., 7-80), lines (e.g., 7-70), and reservoirs to supply the troughs with reagents (e.g., 10 7-40), and a motor (e.g., 2-70) to move troughs 2-20 (See FIGS. 2 and 7).

Each surface upon which the nascent oligomer will be formed is associated with a solenoid capable of independently raising or lowering that surface into a 15 reagent trough. The solenoids and their associated surfaces are arranged in an array above the reagent troughs. For example, the array may comprise a series of rows of solenoids. The troughs can be moved, for example by a stepper motor, to allow any surface access 20 to any trough. It will be understood that a plurality of surfaces may be simultaneously moved, thus allowing multiple synthesis of like or different oligomers.

The operation of synthesizer 1-20 is automatically controlled by a computer device. The 25 computer device may be computer 1-10 or, preferably, a separate computer, microprocessor or microcontroller associated with synthesizer 1-20. The computer device controls the various operations of synthesizer 1-20, and communicates directly with fluid transportation 30 system 1-21 concerning the availability and location of particular synthesized oligomers. If a separate computer device is used, that device receives instructions from computer 1-10 over line 1-12 as to the sequence of the predicted oligomer to be 35 synthesized and informs computer 1-10 over line 1-12 of

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the status of synthesizer 1-20, including the status of synthesis of each predicted oligomer.

As shown in FIG. 2 and described below, the principal components of preferred synthesizer 1-20 are 5 trough module 2-20, reagent reservoir and drive system 2-30, solenoid array 2-40, gastight enclosure 2-50, valve block 2-60, motor 2-70, computer 2-80 and controller 2-90. Each is described in detail below.

As shown in FIG. 3, the trough assembly 10 includes trough module 3-20,\* mounting assembly 3-30, pillow blocks 3-40, travel rails 3-50, drive belt 3-60, pulleys 3-70, stepper motor 3-80, and main drive pulley 3-85.

The trough module, shown in FIG. 4, may be 15 milled from a solid block of polypropylene, although any similar inert material is suitable. In the preferred embodiment, the trough module 4-20 (3-20, 2-20) is 5 inches in length, 1.275 inches deep, and 3.375 inches wide. The troughs 4-30, which are formed 20 in top face 4-25 of trough module 4-20, are numbered 4-12, 4-10, 4-8, 4-4, 4-3, 4-2, 4-1. With the exception of trough 4-8, the troughs are preferably milled to a 60° angle at the base and are 5mm deep. Trough 4-8 may be round bottomed and 8mm deep to facilitate a higher 25 volume of reagent flow during the detritylation step of oligomer synthesis. Troughs 4-30 may be spaced 9mm on-center, so as to be compatible with the spacing found in commercially available 96 well laboratory dishes. Reagent inlet ports 4-40, located on front face 4-45 of 30 trough module 4-20, and reagent delivery lines 4-50 allow delivery of reagents to the troughs 4-30. Reagent inlet ports 4-40 may be tapped for standard

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\* The same element may have different numbers on different figures (e.g., the trough module is 2-20, 3-35 20, 4-20 and 5-20).

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1/4" 28 connections. Reagent exhaust ports 4-60, located on back face 4-65 of trough module 4-20, and reagent exhaust lines 4-70 allow removal of reagents from troughs 4-30. Reagent exhaust ports 4-60 may be 5 tapped for standard 1/4" 28 connections.

As shown in FIG. 4D, bottom surface 4-80 of the reagent inlet and exhaust ports is preferably flat, to allow a good seal with standard 1/4" 28 connectors. All ports and lines are preferably constructed so as to 10 minimize the dead volume of the system while still allowing adequate flow rates. Their surfaces should be smooth to facilitate washing and to minimize contamination.

In the preferred embodiment shown in FIGS. 3, 15 5, and 6, trough module 3-20 is mounted on pillow blocks, (for example TWN-4-ADJ super ball bushing twin pillow block, Atlantic/Tracy, Inc.) to allow travel of trough module 5-20 (3-20) on travel rails 3-50.

Mounting assembly 5-30 (3-30) includes two 1/8 inch 20 aluminum plates 5-35 to which trough module 5-20 (3-20) is rigidly but removably mounted. Pillow blocks 5-40 (3-40) are mounted on the underside of the mounting assembly 5-30 (3-30). Travel rails 3-50 pass through pillow blocks 3-40 (5-40). Drive belt 3-60 is fixed 25 rigidly to drive belt anchor 5-65 and is held in position by pulleys 3-70. Angular displacement of main drive pulley 3-85 by stepper motor 3-80 is translated by drive belt 3-60 into transverse displacement, along travel rails 3-50, of trough module 3-20.

30 Main drive pulley 3-85 is preferably located as close to back plate 5-90 of the gas tight enclosure as possible. Drive belt 3-60 is preferably routed so that it will not interfere with the solenoid array, the reagent supply or exhaust lines connected to the trough 35 unit, or other elements of the device. Drive belt 3-

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60 is positioned as closely to the ends of the gas-tight enclosure as possible and as close to base plate 3-100 (5-100) as possible, and is routed along the centerline of the baseplate 5-100 to trough module 5 5-20. Kill switches (not shown) may be appropriately placed to limit the travel of trough module 3-20 to prevent it from moving into glass endplates 3-110.

The reagent reservoir and drive system provides for the supply and removal of reagents, e.g., 10 monomer, oxidizer, and rinsing agents, to the troughs.

The preferred embodiment is shown in FIG. 7. Reagent reservoir and drive system 7-10 includes gas source 7-20, metering valves 7-30, reagent reservoirs 7-40, 7-42, 7-44 and 7-46, monomer reservoirs 7-50, 7-15 52, 7-54, and 7-56, gas lines 7-60, delivery lines 7-70, two-way valves 7-80, reagent inlet connectors 7-90 (connected to the front face 7-95 of the trough module), reagent exhaust connectors 7-100 (connected to the backface 7-105 of the trough module), exhaust lines 20 7-110, three way valve 7-120, vacuum trap 7-130, and vacuum source 7-140.

In the preferred embodiment, gas pressure from gas source 7-20 provides the motive force for the transfer of reagents from their reservoirs 7-40, 7-42, 25 7-44 and 7-46, through delivery lines 7-70, to reagent inlet connectors 7-90, and thus to the troughs. Motive force for the removal of reagent or monomer from the troughs is provided by vacuum source 7-140. The delivery or removal of a reagent is controlled by 30 opening and closing appropriate subsets of valves 7-80 or 7-120. The operation of valves 7-80 and 7-120 is controlled by computer. An example of such control is described below.

Argon is a suitable inert gas (although other 35 inert gases may also be suitable) for use in the

reagent reservoir and drive system 7-10. A standard 2-stage regulator may be used to release ultra-pure argon from the supply tank, for instance, at approximately 10 psi. The lines supplying argon to the 5 monomer reservoirs may be set at 6 psi. The monomers and tetrazole may be delivered from the bottles they are shipped in. Argon pressure may be delivered to each bottle through a 20 gauge 1 1/2" needle attached to a male luer (w/lock)-1/4" 24 adaptor connected to 10 the appropriate general valve connector. The other reagents are preferably stored in separate reservoirs, for instance, Kontes HPLC/synthesizer reservoirs. Argon may be delivered to these separate reservoirs at, for instance, 3 psi directly to inlet ports in the caps 15 of the reservoirs.

The preferred embodiment includes a solenoid-piston-reagent-pin assembly 8-10, as shown in FIG. 8. The assembly includes solenoid 8-20, piston 8-30, retaining ring 8-40, connecting rod 8-50, adaptor 8-60, 20 pin socket 8-70, pin 8-80, and reagent tip 8-90.

A Guardian T 3.5 X 9-C12D, 12 volt DC continuous duty tubular solenoid is a suitable solenoid. Retaining ring 8-40 may be fabricated from polypropylene, e.g., a one millimeter thick slice cut 25 from a Rainin RT96 pipette tip or any other suitable material. Connecting rod 8-50 is a shaft, preferably plastic, which can be fabricated, for example, from the positive displacement piston of a Gilson CP-250 pipette. Adaptor 8-60 may be fabricated from a piece 30 of flexible tubing, for example, Cole-Parmer 1/32 X 3/32 C-flex tubing. Pin socket 8-70 may be fabricated from the distal six millimeters of a Rainin RT96 pipette tip. Pin 8-80 may be a steel rod and can be 35 fabricated from the displacement piston of a Gilson CP-50 pipette. Reagent tip 8-90 is preferably made of

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polypropylene. The proximal fitting from the steel displacement piston of a Gilson CP-50 pipette may be used for a reagent tip.

Assemblies 8-10 are mounted in solenoid array 5 frame 8-100. Frame 8-100 may be fabricated from a Rainin RT96 pipette box. Assemblies 8-10 can be positioned in an array such that the longitudinal axis of the pistons are aligned with the spacing found in standard 96-well titre plates. Solenoids 8-20 are 10 preferably positioned in a staggered, two tier, configuration, because the solenoid diameter is too great to allow them to be packed adjacently, i.e., at the same level. (See FIG. 13). The two tier arrangement also allows improved heat dissipation.

15 In the preferred embodiment, piston 8-30 is inserted into solenoid 8-20. Retaining ring 8-40 is fitted onto piston 8-30. Connecting rod 8-50 links adapter 8-60 to piston 8-30 and on the other end to pin socket 8-70. Pin 8-80 fits removably into pin socket 20 8-70. Retaining ring 8-40 prevents the piston from seating fully on energizing of solenoid 8-20. If allowed to seat fully residual magnetism prevents the piston from descending when the power is removed. Beneath the piston assembly, upper guide plate 8-110 25 and lower guide plate 8-120 align reagent pin 8-80 and direct it to the trough. Guideplates 8-110 and 8-120 are fixed rigidly with respect to solenoid array frame 8-100. A clearance of about 1mm exists between tip 8-90 and the top surface of the trough when the solenoids 30 are energized. Upper guide plate 8-110 prevents the piston from falling out of the solenoid and determines the magnitude of piston travel. Total piston travel is about 8mm.

Oligomers are synthesized on a solid phase 35 substrate surface adhered to the reagent tips. Any

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suitable solid phase substrate surface, including glass fiber, cellulose, or controlled pore glass beads, can be used to form the surface. Commercially available controlled pore derivatized beads to which A, T, G, or 5 C monomer has been attached such as used in column based DNA synthesis are particularly convenient for use with the synthesizer. Examples include those available from ABI with a controlled pore glass ABI part number 400386, C controlled pore glass ABI part number 300387, 10 G controlled pore glass ABI part number 400388, and T controlled pore glass ABI part number 400389 or from Milligen.

The beads may be adhered to the reagent tip by heating the reagent tip until it is just molten, 15 then forcing the heated tip into a shallow container filled with the appropriate bead. As understood by those skilled in the art, when beads coupled to a given monomer are used to synthesize a surface, that monomer will provide the first nucleotide, in the 5' to 3' 20 direction, of the oligomer formed on that surface.

Gas-tight enclosure 2-50 provides a solid base on which other components, e.g., motor 2-70, travel rails 3-50, and solenoid array 2-40 can be mounted. Gas-tight enclosure 2-50 also isolates 25 reagent tips 8-90 and reagents from the atmosphere.

The entire enclosure may be purged of atmospheric gases by argon delivered through a regulator.

In the preferred embodiment (FIGS. 2, 3 and 30 5), gas-tight enclosure 2-50 has front plate 5-88, back plate 5-90, and base plate 5-100, which may be fabricated from 0.3125" aluminum, and endplates 3-110 and top plate 3-120 which may be fabricated from 0.3125" glass. Silicone gaskets are glued to the glass 35 plates, and the glass plates clamped to the aluminum

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structure to provide a gas-tight environment. A humidity meter (not shown) may be attached to top plate 3-120 with its probe snaked between the aluminum side and top silicone seal. Lines 7-70 delivering fresh 5 reagent enter the device through the front plate 5-88. Exhaust lines 7-110, argon purge lines (not shown), and electrical connections, pass through back plate 5-90. Motor 2-70 is attached to back plate 5-90. Trough module 3-20 travels on rails 3-50 that are attached to 10 base plate 3-100 (5-100). Solenoid array 3-125 (5-125) rests on supports that are connected to front plate 5-88 and back plate 5-90. Solenoid array 3-125 (5-125) is firmly seated yet removable.

The valve block provides valves that control 15 the flow of reagents to and from the reagent troughs.

In the preferred embodiment, the valve block consists of 24 12VDC 2-way teflon valves 7-80 (e.g., GV#2-17-900, General Valve Corp.) that control the flow of monomers and other agents to and from the trough 20 module and 3-way teflon valve 7-120 CA (e.g., GV#1-17-900 General Valve Corp.) that controls exhaust vacuum. 2-way valves 7-80 are closed when not energized and open when energized. All 2-way exhaust valves 7-80, (e.g., Omega relay # 7-12, see below) are preferably 25 connected to 3-way valve 7-120 such that the main force of the vacuum is isolated from 2-way valves 7-80 when 2-way valves 7-80 are closed. The vacuum valves 7-80 and 7-120 may be electrically isolated from each other via diodes.

30 The valves may be mounted on Rainin RT96 pipet tip racks and housed in Rainin TR96 pipet tip boxes. All non-monomer connections may be made with 1/16" x 1/32" teflon tubing and the appropriate fittings supplied by, for example, General Valve 35 according to their recommendations. The monomers and

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tetrazole lines may consist of 0.007" ID tubing. Although small-bore tubing is desirable in that it minimizes dead volume, the additional trough-filling time required by small bore tubing may be undesirable.

5 A Super Vexta PH268M-E1.5B 2-phase stepper motor, available from Inductive Components, is a suitable motor for moving trough module 3-20.

If the synthesizer is controlled by a separate computer device, either Digital Microvax or 10 Radio Shack TRS 80 Model 100 computers are suitable. Instructions from computer 1-10, or the separate computer device, determine the sequence in which the reagents come into contact with each surface and thus determine the nucleotide sequence of the oligomer 15 synthesized on each surface. Such instructions include instructions: to open or to close various valves, to introduce or to remove a reagent from a trough, to move the troughs, or to raise or to lower a given surface. The instructions are implemented by an interface.

20 A suitable interface is Omega OM900 Series Interface (Omega Instruments, Stamford, Connecticut) or similar device capable of converting instructions from computer 1-10, or a separate computer device, into signals that can control the electro-mechanical devices 25 of synthesizer 1-20. The Omega OM900 Series interface includes a central processing unit (OM992 Central Processing Unit), an interface power supply (OM-903 Power Supply), and a multiplex module (OM915 Multiplex Module) with 32 individually addressable relays which 30 are used to control synthesizer 1-20. For example, if six solenoids are included in synthesizer 1-20, the 32 relays may be assigned as shown in Table 1.

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Table 1

<u>Relay Number</u>	<u>Function controlled</u>
1	Oxidizer reagent (tert-butyl hydroperoxide (tBHP) delivery valve
2	Detritylation reagent (trichloroacetic acid (TCA)) delivery valve
3	Adenine (B, dA cyanoethyl phosphoramidite) delivery valve
3	Tetrazole delivery valve
4	Cytosine (B, dC cyanoethyl phosphoramidite) delivery valve
4	Tetrazole delivery valve
5	Guanine (iBu dG cyanoethyl phosphoramidite) delivery valve
5	Tetrazole delivery valve
6	Thymine (T cyanoethyl phosphoramidite) delivery valve
6	Tetrazole delivery valve
7	Oxidizer (tBHP) reagent exhaust valve
8	Detritylation (TCP) reagent exhaust valve
9	Adenine exhaust valve
10	Cytosine exhaust valve
11	Guanine exhaust valve
12	Thymine exhaust valve
13	Oxidizer rinse delivery valve
14	Detritylation rinse delivery valve

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- 15 Adenine rinse delivery valve
- 16 Cytosine rinse delivery valve
- 17 Guanine rinse delivery valve
- 18 Thymine rinse delivery valve
- 19 Solenoid No. 1 (initial activation),  
12 VDC supply
- 20 Solenoid No. 2 (initial activation),  
12 VDC supply
- 21 Solenoid No. 3 (initial activation),  
12 VDC supply
- 22 Solenoid No. 4 (initial activation),  
12 VDC supply
- 23 Solenoid No. 5 (initial activation),  
12 VDC supply
- 24 Solenoid No. 6 (initial activation),  
12 VDC supply
- 25 Stepper motor (motor drive) 5 VDC supply
- 26 Stepper motor (motor direction) 5 VDC  
supply
- 27 Solenoid No. 1 (maintenance), 5 VDC  
supply
- 28 Solenoid No. 2 (maintenance), 5 VDC  
supply
- 29 Solenoid No. 3 (maintenance), 5 VDC  
supply
- 30 Solenoid No. 4 (maintenance), 5 VDC  
supply
- 31 Solenoid No. 5 (maintenance), 5 VDC  
supply
- 32 Solenoid No. 6 (maintenance), 5 VDC  
supply

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Solenoids Nos. 1 to 6, described in Table 1 above, are each associated with a corresponding solenoid-piston-reagent-pin assembly.

For such a configuration, it will be apparent

- 5 that Relay No. 1 opens a valve which allows oxidizer to flow into the appropriate trough (trough 4-12); Relay No. 3 opens two valves, one which allows adenine monomer, and one which allows tetrazole to flow into the appropriate trough (trough 4-4); Relay No. 7 opens
- 10 a valve which allows the contents to be removed from the oxidizer trough (trough 4-12); Relay No. 13 opens a valve which allows a rinsing solution to flow into the oxidizer trough (trough 4-12); Relay No. 19 controls the 12 VDC supply to solenoid No. 1 (12 VDC is required
- 15 to initially activate the solenoid); Relay No. 27 controls the supply of 5VDC to solenoid No. 1 (the voltage required to maintain the solenoid in the activated state); and Relay Nos. 25 and 26 which control respectively, the power to the stepper motor
- 20 and the direction of the stepper motor, control the positioning of the trough module.

The assignment of a single relay to control both monomer and tetrazole valves (relays 3 to 6) is due to constraints imposed by the number of relays on

- 25 the OM-915. Increasing the number of relays would remove the need to place more than one valve on a relay. More importantly, increasing the number of relays would allow an increase in the number oligomers that can be simultaneously synthesized. For example,
- 30 the system could easily be expanded to seven more OM-915 units. In such a configuration, one OM-915 unit would be dedicated to valve and motor control and the other OM-915 units would be dedicated to solenoid control. The use of seven OM-915 units thus configured

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would allow 96 controllable solenoids, and thus allow the synthesis of 96 oligomers simultaneously.

FIG. 9 depicts the terminal board 9-20 of the OM-915 interface and associated connectors. Relay 5 terminals 9-30 are labeled with their relay numbers. Power is supplied to relays 9-30, in banks of four relays, (e.g., Relay Nos. 1 to 4 constitute a bank, and Relay Nos. 5 to 8, constitute a separate bank), by common contact (labeled c) and ground contact (labeled 10 g). Relay Nos. 1 to 24, which control valves, are supplied with the 12 VDC from 12 VDC source 9-60. Relay Nos. 25 and 26, which control the motor, are supplied with 5 VDC from 5 VDC source 9-70. Relay Nos. 27 to 32, which control the activation and 15 maintenance of the energization of the solenoids, are supplied with 12 VDC (for initial activation) from Relay Nos. 19 to 24, and 5 VDC (for maintenance of activation) from the 5 VDC source 70. A SOLV 30-12 12 VDC 4A power supply (Newark Electronics), is a suitable 20 12 VDC source and an Elpac Model WM113+12/-12/+5 VDC power supply is a suitable 5 VDC source.

Relays controlling the valves are connected to controller-side valve block connector 9-80. As described in Table 1, Relay Nos. 3, 4, 5, and 6 each 25 controls two valves. Each of these relays is thus connected to two pins on controller-side valve block connector 9-80. Controller-side valve block connector 9-80 is connected to machine-side valve block connector 10-20 shown in FIG. 10 which depicts the 30 wiring of the valve block.

Machine-side valve block connector 10-20 may be connected by lines 10-30 to two-way valve controlling solenoids 10-40 to 10-86 and three-way valve controlling solenoid 10-90. The valve solenoids

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may be electrically isolated by diodes 10-50 or light emitting diodes 10-100.

Activation of any of valve controlling solenoids 10-40, 10-42, 10-44, 10-46, 10-48, or 10-50,

- 5 which are the valves controlling exhaust of a reagent from a trough, also preferably activates 3-way valve controlling solenoid 10-90. For example, activation of valve controlling solenoid 10-40 by a signal transmitted on line 10-30a also activates the 3-way
- 10 valve controlling solenoid 10-90. Thus, both the exhaust valve and vacuum control valve are opened, allowing vacuum to exhaust the trough. The circled numbers in FIG. 10 indicate the number associated with the 2-way valve controlled by a solenoid.

- 15 As shown in FIGS. 9 and 11, Relay Nos. 25 to 26, which control stepper motor 11-60, are preferably connected directly to stepper motor controller 9-140 by lines 11-160. 120 VAC source 9-150 is also connected to stepper motor controller 9-140. Lines 9-160 connect
- 20 stepper motor controller 9-140, by way of controller-side enclosure connector 9-90, machine-side enclosure connector 10-20, and lines 9-160, to stepper motor 11-60. Relay Nos. 19 to 24 and 27 to 32, which control the solenoids, are connected by lines 9-120 to
- 25 controller-side enclosure connector 9-90. Controller-side enclosure connector 9-90 connects to machine-side enclosure connector 11-20 shown in FIG. 11. Machine-side enclosure connector 11-20 is connected to solenoids 11-40, which may be organized in a 2 x 3
- 30 array, by lines 11-50.

With reference to Table 1, and FIGS. 9 and 11, a solenoid is preferably activated as follows. A solenoid is lifted by 12 VDC. For example, a signal from the computer closes Relay No. 19, supplying 12 VDC

- 35 by line 9-130 to Relay No. 27. Relay No. 27 in turn

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supplies 12 VDC by line 9-120a to controller-side enclosure controller 9-90, to machine-side enclosure 11-20, to line 11-50a and, to hence, to solenoid 1.

After being lifted, the solenoid may be

5 maintained in the activated state by 5 VDC. The computer instructs Relay No. 27 to close, supplying 5 VDC to solenoid 1 and opens Relay No. 19, to remove the 12 VDC potential used to initially activate solenoid 1. At another signal from the computer, Relay No. 27 opens  
10 and the 5 VDC potential is cut off.

The simultaneous synthesis of multiple oligomers of any chosen sequence is directed by computer 1-10. The positioning of the troughs, movement of the surfaces, and the filling and emptying  
15 of the troughs may be controlled by computer 1-10 alone or, preferably, by a separate computer system associated with synthesizer 1-20.

For example, where a separate computer system is used, it may operate using a Fortran program  
20 (Appendix A, attached).

The general operation of a suitable program is shown in FIG. 12. As shown in FIG. 12A the program first initializes the system (step 12-20). Initialization may include filling the reagent troughs,  
25 bringing selected monomer troughs into position under appropriate solenoids, then dipping the surfaces into any trough required for activation.

A monomer is then added (step 12-30), the growing molecules are capped (step 12-40), oxidized  
30 (step 12-50) and deprotected (step 12-60). If there are no more additions, the program ends 12-70. If there are more monomers to be added (step 12-80), the program returns to add the next monomer (step 12-30).

The process for the addition of a monomer is  
35 described in more detail in FIG. 12B. The process for

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capping, oxidation, and detritylation is described in more detail in FIG. 11C.

The program may also include a subroutine that, upon entry of the predicted sequences, determines 5 the optimum assignment of sequences to surfaces; determines the optimum order of the placement of reagent troughs; or controls post-synthesis modification, such as elution or washing.

The chemistry of DNA synthesis is well known 10 to those skilled in the art, see e.g., Froeler et al. 1988, Nuc. Acid. Res. 14:5399-5407, hereby incorporated by reference. The phosphoramidite method, see Matteucci et al., 1981, J. Am. Chem. Soc. 103:3185-3191, hereby incorporated by reference and Caruthers 15 et al., 1987, Methods Enzym. 154:287-313, hereby incorporated by reference, is suitable for use with synthesizer 20 and is described briefly below.

In the phosphoramidite method, 5' protected monomers are added to the 5' OH group on the growing 20 molecule chain. Addition of a monomer subunit to the growing nucleotide chain requires the following steps: (1) removal of the dimethoxytrityl protecting group from the previously added (or starting) monomer with trichloroacetic acid (TCA) to form a reactive 5' 25 hydroxyl group; (2) the addition of a 5' dimethoxytrityl protected monomer by condensation; (3) acetylation or capping of the unreactive deoxynucleoside (Step 3 is optional); and (4) oxidation of the phosphite triester to the phosphate triester 30 with tert-butyl hydroperoxide or iodine/water.

Synthesis proceeds stepwise in a 5' to 3' direction by the sequential addition of monomers.

Oligomers are synthesized on the surfaces that are adhered to the reagent tips. The reactions 35 needed to add a monomer to the nascent oligomer

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synthesized on a surface are effected by:

(1) utilizing troughs filled with the appropriate reagents, such as a monomer, oxidizer, or rinsing agent, (2) positioning the appropriate troughs beneath 5 the appropriate surfaces in an appropriate order, and (3) lowering the surface into the trough. For example, addition of a monomer to a growing chain on a surface requires the following steps: (1) activating the previously added monomer by positioning the trough 10 filled with detritylation reagent below the surface, dipping the surface into the detritylation reagent to remove the dimethoxyl-trityl protecting group, removing the detritylation reagent from the trough, filling the trough with a rinsing agent such as, acetonitrile to 15 rinse the trough and surface, and lifting the surface; (2) adding a protected monomer by repositioning a trough containing the appropriate monomer below the surface, dipping the surface into the monomer trough, removing the monomer reagent from the trough, filling 20 the trough with rinsing agent to rinse the trough and surface, and lifting the surface; and (3) oxidizing the phosphite triester bond by positioning a trough containing the oxidizer, such as tert-butyl hydroperoxide under the surface, dipping the surface 25 into the oxidizer to oxidize the phosphite triester bond to a phosphate triester, removing the oxidizer and filling the trough with rinsing agent to rinse the trough and surface, and lifting the surface from the trough. This sequence of reactions results in the 30 addition of a protected monomer to the oligomers being synthesized on the surface.

In the specific and particular embodiment described herein, reagents, reaction times, and trough designations are as follows. Adenine monomer reagent 35 consists of one gram of Bz dA cyanoethyl

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phosphoramidite in 12 ml anhydrous acetonitrile and occupies trough No. 4-4, thymine monomer reagent consists of one gram of T cyanoethyl phosphoramidite in 12 ml anhydrous acetonitrile and occupies trough

- 5 No. 4-1, cytosine monomer reagent consists of one gram of B2 dC cyanoethyl phosphoramidite in 12 ml anhydrous acetonitrile and occupies trough No. 4-3, and guanine monomer reagent consists of one gram of iBu dG cyanoethyl phosphoramidite in 12 ml anhydrous acetonitrile and occupies trough No. 4-2. Surfaces are exposed to monomer for three minutes then washed in acetonitrile for one minute. It should, however, be understood that other concentrations, times, washes, washing schedules and trough arrangements are within
- 10 the scope of this invention.

Detritylation reagent consists of trichloroacetic acid in dichloromethane and occupies trough No. 4-8. Surfaces are held in the detritylation reagent for three minutes. The rinsing agent which follows detritylation consists of acetonitrile. Surfaces are rinsed for one minute. The oxidizing reagent consists of tert-butyl hydroperoxide 1.1 M in anhydrous dichloromethane and occupies trough No. 4-12. Surfaces are held in the oxidizing reagent for one

- 25 minute: After oxidation, the surfaces and troughs are washed in acetonitrile for one minute.

Rinsing normally occurs in the same trough in which the previous reaction occurred, e.g., the post tritylation rinse occurs in trough 4-8.

- 30 At the beginning of the run, the enclosure and all lines are purged with argon and all troughs are filled with the appropriate reagents. All surfaces are lifted and the trough module is moved to the initiation position. The initiation position places trough 4-8
- 35 (TCA) beneath the designated solenoid.

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For the sake of simplicity, the action of a single surface has been described above, in actual operation, synthesis of multiple oligomers will proceed simultaneously on multiple surfaces. This may be 5 accomplished by positioning the solenoids in an array, for instance, an array of rows. For example, as shown in FIG. 13 the solenoids 13-20a, 13-20b, 13-20c, in a given row 13-20a are aligned such that the surfaces can all be dipped into trough 13-30 positioned under that 10 row of solenoids. When a trough is positioned under a row, all of the surfaces in that row that require contact with the reagent in the trough may be lowered simultaneously. Surfaces that do not require contact with the reagent are not lowered. While surfaces in a 15 row above a given trough are being lowered, the solenoids in the adjacent rows in either direction can be lowered in the corresponding adjacent troughs. Thus, synthesis of the oligomers on all surfaces is truly simultaneous.

20 In addition to synthesizing oligomers, synthesizer 1-20 performs other steps necessary to prepare the synthesized oligomers for use in hybridization. These steps include eluting the oligomers from the surfaces on reagent tips 8-90, 25 purifying the eluted oligomers, labelling the purified oligomers, and placing each labelled oligomer in transportation system 1-21 to be sent to the corresponding selected membrane unit 14-50 (15-50, 16-50, 17-50 and 19-50) contained in membrane unit array 30 1-30 for hybridization to nucleic acid sequencing patterns bound to membrane 16-51.

It will be obvious to one skilled in the art that the composition of the eluting solution will depend on the mode of synthesis of the oligomers. For 35 example, if conventional oligonucleotide synthesis

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involving addition of acetate caps onto unreacted nucleotide chains is employed, the longest resulting oligomer will contain a trityl group on the 5' end. Such an oligomer may be eluted and deblocked in one 5 step, for example, by heating in a solution of NH<sub>4</sub>OH contained within the well of a 96 well plate. After evaporation of the NH<sub>4</sub>OH, the oligomers may be labelled directly in the well. The oligomers may then be transferred from the well by fluid transportation 10 system 1-21 to membrane unit array 1-30.

Alternatively, the oligomers may, if desired, be purified to eliminate all but the longest nucleotide chains. Purification may be performed by eluting the oligomers with methanol and automatically transferring 15 the eluted oligomers contained in the wells to a corresponding number of affinity columns, each containing a substance, such as oligopak or Nensorb, that binds the trityl group of the oligomer. Such chromatography thereby removes any capped failure 20 molecules. The purified oligomers are then eluted from the columns into the wells of another disposable multi-well plate.

The oligomers may be labelled in the wells by one of a variety of methods to permit their detection 25 after hybridization to sequencing patterns bound to membrane 16-51. Any of a number of labelling methods may be employed, including those employing radioactive labels [T. Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, 30 1982], fluorescent [(L.M. Smith et al., *Nuc. Acid. Res.*, 13, pp. 2399-412 (1985); J.M. Prober et al., *Science*, 238, pp. 336-41 (1987); W. Ansorge, et al., *Nuc. Acid. Res.*, 15, 4593-603 (1987); J.A. Brumbaugh, et al., *Proc. Natl. Acad. Sci. USA*, 85, pp. 5610-14 35 (1988)], calorimetric [(S. Beck, *Anal. Biochem.*, 164,

pp. 514-20 (1987); P. Richterich, et al. Biotechniques, 7, pp. 52-59 (1989)], and chemiluminescent labels [S. Beck, et al., Nuc. Acid. Res., 17, pp. 5115-122 (1989)]. As will be understood by one skilled in the art, the method of labelling chosen must conform to the type of detector 1-40 employed. After labelling, any unincorporated label may be removed if desired, by passing the labeled oligomer over a sizing column. It is preferred that the labelling be conducted by a method that does not generate a significant amount of unincorporated label. The labelled oligomers are then introduced into fluid transportation system 1-21, described below, which connects synthesizer 1-20 to each membrane unit 1-50 contained in membrane unit array 1-30.

The above described mechanical steps performed by synthesizer 1-20 after oligomers have been synthesized can be automated using any of a number of commercially available devices, with minor modifications. For example, robots such as the Xymark free-hand robot, and machines capable of handling the multiple-well plates and transferring materials to and from the plates, such as those sold by Biomek and Lab Systems, are readily available for use with synthesizer 1-20.

Membrane unit array 1-30, which is shown generally in FIGS. 14 and 15, contains a plurality of membrane unit stations 14-32, each of which holds a membrane unit 14-50. Stations 14-32 are separately connected to transportation system 1-21, enabling fluids to be independently supplied to and removed from each membrane unit 14-50. Array 1-30 may contain any number of membrane units 14-50, with a corresponding number of membrane unit stations 14-32. For example, array 1-30 may contain 400 membrane units 14-50 and

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stations 14-32. It is apparent that array 1-30 of such size has substantially more stations 15-32 than are shown generally in FIGS. 14 and 15.

Each membrane unit 16-50 (14-50) includes a 5 membrane 16-51, upon which, prior to automated operation of sequencer 1-1, nucleic acid sequencing patterns have been immobilized.

In the preferred embodiment, the patterns bound to membrane 16-51 are multiplex sequencing 10 patterns, i.e., multiple, overlayed, nucleic acid sequence patterns capable of being repeatedly hybridized with different probes to reveal different nucleic acid sequence ladders (see Ohara et al, supra).

The DNA used to generate the multiplex 15 sequencing patterns may be cloned. Alternatively, genomic DNA may be used directly. When using the method and apparatus of the present invention to sequence DNA from higher organisms, for instance chromosomes with the complexity of mammalian DNA, it 20 may be useful to first clone the DNA into vectors such as yeast artificial chromosomes. Genetic manipulation of yeast artificial chromosomes is well known to those skilled in the art.

Any procedure that cleaves DNA at defined 25 locations and results in well defined 5' and 3' ends may be used to fragment the DNA prior to the sequencing reactions. In the preferred embodiment, a multiplex sequencing pattern is generated from chemical cleavage of DNA fragmented by restriction enzyme digestion. The 30 DNA is first digested to completion with different restriction enzymes or combinations of enzymes, such that the resulting DNA fragments are of a given average size, yet have many different 5' and 3' ends. For example, enzymes that recognize a sequence of 6 bases 35 cut DNA an average of once per 4000 base pairs.

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Digestions with combinations of such enzymes produce smaller fragments. Varying the combinations results in variation of the 5' and 3' ends of the fragments.

When gels that allow reading of a sequence up to approximately 300 base pairs are utilized, it is preferred that the DNA be digested with combinations of enzymes to produce fragments of 700 to 800 base pairs. When longer gels are utilized, for example gels that allow reading of sequence up to 600 bases, it may be advantageous to choose a combination of enzymes that will result in the DNA being cleaved on average every 1000 base pairs. Alternatively, digestion may be performed with multiple single enzymes that recognize a sequence of fewer bases.

After digestion, each sample is subjected to sequencing reactions to generate multiplex sequencing patterns. For example, chemical cleavage reactions such as the five standard reactions [A.M. Maxam and W. Gilbert, "Sequencing End-Labeled DNA with Base-Specific Chemical Cleavages", Meth. Enzym., 65, pp. 499-560 (1980)] may be employed. Modifications of the standard sequencing reactions, for instance those described in United States Patent No. 4,865,968, may also be employed. Alternatively, the multiplex sequencing patterns may be generated by enzymatic synthesis. It is preferred that 1 to 30 micrograms of DNA be employed for each sequencing reaction.

Sequencing reactions derived from a multiplicity of restriction digested DNA samples may then be loaded onto the same gel for subsequent electrophoresis. For example, a single gel may contain the sequencing products of 18 or more single or combination digests.

The products of the sequencing reactions may be resolved electrophoretically to form numerous

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overlaid nucleic acid sequence ladders, i.e., multiplex sequencing patterns. At present, denaturing gels of 5-20% acrylamide are commonly used for electrophoretic separation. Such gels may be wedge 5 shaped or of uniform thickness, and may be run using a variety of buffer systems and electrophoretic conditions well known to those skilled in the art.

After electrophoresis, the multiplex sequencing patterns are transferred onto membrane 10 16-51. Such transfer may be accomplished by any of a variety of methods. For example, the patterns may be electrotransferred after electrophoresis as described in G.M. Church and W. Gilbert, supra. Alternatively, the DNA may be transferred during electrophoresis in 15 the method described by S. Beck and F.M. Pohl, "DNA Sequencing With Direct Blotting Electrophoresis", EMBO J., 3, pp. 2905-2909 (1984). A variety of membranes may be employed in the present invention, for example DBM, DPT, and polyvinylidene fluoride membranes. Nylon 20 membranes are preferred, as they are able to withstand vigorous washing.

After transfer, the sequencing patterns must be immobilized on the membrane such that they will remain bonded throughout repeated cycles of 25 hybridization, washing and stripping. It is preferred that the sequencing patterns are covalently bound to the membrane by UV crosslinking, for instance as described in G.M. Church and W. Gilbert, supra. Other techniques of linking nucleic acids to membranes may 30 also be useful and are well known to those skilled in the art.

After binding is complete, each membrane 16-51 is placed in a separate membrane unit 16-50. Membrane unit 16-50 is designed to permit 35 several operations to be repeatedly performed on

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membrane 16-51, including hybridization of labelled oligomers to the sequencing patterns bound to membrane 16-51, washing of membrane 16-51, movement of hybridized membrane 16-51 to be detected by detector 5 15-40, and stripping of membrane 16-51.

The elements of the preferred embodiment of membrane unit 16-50 are shown in FIG. 16. Membrane 16-51 is bonded to a face of backing plate 16-52 within the margins of plate 16-52. Plate 16-52 may be made of 10 a number of materials including glass or plastic.

Flexible sheet 16-53, preferably made of plastic, covers the face of plate 16-52 on which membrane 16-51 is bonded, and is itself bonded to plate 16-52 near the margins of plate 16-52, enclosing a space between sheet 15 16-53 and membrane 16-51. Flexible sheet 16-53 has at least one, and preferably a series of inlet and outlet tubes 16-54 formed therethrough. The free end of each tube 16-54 is connected to fluid transportation system 1-21 within station 14-32 to supply membrane unit 14- 20 50 (16-50) with fluids and to drain fluids from membrane unit 16-50 for disposal.

Preparation of membranes 16-51 and assembly of membrane units 16-50 preferably occurs prior to the commencement of automated operation of sequencer 1-1. 25 Membranes 16-51 and membrane units 16-50 may be changed, substituted or reassembled during operation of sequencer 1-1.

It will be understood that bonded membrane 16-51 is reusable many times, at least 50 times and 30 probably several hundred times, because the bonding eliminates most of the current sources of failure that are produced through flexing of membranes during hybridization, washing and stripping. By reducing or eliminating the time required to replace failed 35 membranes, the speed of sequencing is much enhanced.

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In order for hybridization to occur properly, it is required that flexible sheet 16-53 be separated from membrane 16-51 a specified distance, with fluid contained in the enclosed space between flexible sheet 5 16-53 and membrane 16-51. It is preferred that the fluid flow between membrane 16-51 and flexible sheet 16-53 be in a 100-300 micron layer.

Cover plate 16-55, shown in FIG. 16, is provided for each membrane unit 16-50 to maintain the 10 desired separation between sheet 16-53 and membrane 16-51. Cover plate 16-55 opposes the side of sheet 16-53 facing away from membrane 16-51. The operation of membrane unit 16-50 in conjunction with cover plate 16-55 is depicted in FIGS. 17a and 17b. Cover plate 16-55 (16-55) moves toward membrane unit 17-50 (16-50) from the released position shown in FIG. 17a to the compressed position shown in FIG. 17b. Alternatively, cover plate 17-55 can be fixed and membrane unit 17-50 may be moved, or both cover plate 17-55 and membrane 15 unit 17-50 can be moved. In the compressed position, cover plate 17-55 contacts and compresses covering sheet 17-53 to limit pressurized fluid flow to a layer of desired thickness. When so compressed, hybridization, washing and stripping occur.

25 The movement of cover plate 17-55 between a released position and a compressed position can be achieved by any of a number of well-known devices, and is automatically achieved through computer control. Although the various operations associated with 30 hybridizing, washing and stripping membrane 17-51 within membrane unit 17-50 are ultimately controlled under direction of computer 1-10, another computer, microprocessor or microcontroller associated with array 1-30 may further direct and control the specific 35 operations that are performed in array 1-30.

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Communication between array 1-30 and computer 1-10, or between a separate computer device associated with array 1-30 and computer 1-10, occurs over line 1-13. Such communication from computer 1-10 informs array

5 1-30 of the selected membrane unit that each synthesized oligomer will be directed to, and prepares that unit for hybridization. In return, computer 1-10 is informed of the status and availability of each membrane unit contained in array 1-30.

10 Optimal performance conditions may require regulation of the temperature of the pressurized fluid. Accordingly, in the preferred embodiment, cover plate 17-55 also contains a means to regulate the temperature of the fluid flowing over membrane 17-51. This means  
15 is preferably a Peltier effect, but could also be achieved by fluid flow in coils embedded in or backing plate 17-55, where plate 17-55 is made of a thermally conductive material such as a metal. The regulation of temperature is controlled by the separate computer  
20 device associated with membrane unit array 1-30 under the direction of computer 1-10, or directly by computer 1-10.

Membrane unit array 1-30 may be of arbitrary size. For example, it may contain 100 units, or more  
25 than 1000 units. Each membrane unit 1-50 operates independently of the other units, however. It will thus be understood that, at any given time, any hybridizing, washing or stripping step, or any stage of such step may occur within any membrane unit 17-50.  
30 Accordingly, the hybridization process may proceed simultaneously within different membrane units 17-50. Moreover, different oligomers may be used for hybridization within different units 17-50. Computer control of the operations performed within array 1-30  
35 permits automatic, overlapping repetition of these

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operations within simultaneously used membrane units 17-50. As a result, newly hybridized membranes 17-51 are produced more frequently for detection, and the overall speed of sequencing is increased.

5        The hybridization of labelled oligomers to the sequencing patterns bound to membrane 17-51 is achieved by flowing a series of fluids over membrane 17-51 that enable hybridization, washing and stripping of membrane 17-51. Such use of solutions is known in  
10      the art, as described, for example, in United States Patent 4,942,124.

Transportation system 1-21 is provided to transport fluids to and from membrane 17-51 under the force of fluid or air pressure through a series of  
15      tubing and valves connected to inlet and outlet tubes 17-54. System 1-21 is separately connected to each membrane unit 17-50 within stations 15-32. Each membrane unit may have a set of computer actuated valves, one for each tube 16-54, for controlling the  
20      flow of fluid contained in system 1-21. Similarly, a separate tube and associated valve may be provided for each fluid contained in system 1-21. Alternatively, several membrane units may be linked on a common input line to all fluids, with each membrane unit having a  
25      single valve connection to the end of the input line and each fluid source having a single valve connection to the start of the input line. FIG. 18 generally shows system 1-21 with a common linkage, and, for simplicity, shows the connection of system 1-21 to only  
30      two membrane units 18-50 and a membrane 18-51.

System 1-21 also has an associated waste fluid disposal system. Outlet tube 16-54 of each membrane unit is connected to one or more waste reservoirs by means of a waste valve and associated  
35      tubing. System 1-21 may be configured so that a single

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tube 16-54 is used for both inlet and outlet purposes, depending upon the combination of opened inlet and outlet valves of system 1-21.

System 1-21 is also connected to synthesizer

- 5 1-20, enabling any synthesized oligomer to be transported to any membrane 16-51 in accordance with the selection determined by computer 1-10. The transportation of the oligomer to a selected membrane 16-51 is ultimately controlled by computer 1-10.
- 10 However, one or more additional computer devices associated with synthesizer 1-20 or membrane unit array 1-30 may also be employed to control operation of system 1-21. The computer device controls temperature, liquid, air and vacuum flow through the valve system
- 15 associated with system 1-21.

After hybridization and washing within a membrane unit 16-50 is complete, that membrane unit 16-50 is presented to detector 15-40 to detect the patterns that result from the hybridization of labelled oligomers to the sequencing patterns bound to membrane 16-51.

In one embodiment of detector 1-40, detector element 19-56 is used to record the patterns produced by hybridization of radioactively labelled oligomers.

- 25 The operation of membrane unit 15-50 (16-50) in conjunction with that embodiment of detector 1-40 is shown in FIGS. 19a and 19b. As seen in FIGS. 19a and 19b, cover plate 19-55 is retracted to permit insertion of detector element 19-56 between membrane unit 19-50 (16-50) and plate 19-55 (16-55). Detector element 19-56 in this embodiment may be film, or a Beta-ray detection sheet, for example.
- 30
- 35

Element 19-56 is first placed over cover sheet 19-53. Plate 19-55 is then moved to the compressed position to place detector element 19-56 in

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contact with membrane unit 19-50, as shown in FIG. 19b. Contact is maintained until an image of the patterns is produced on element 19-56. Plate 19-55 is then retracted from membrane unit 19-50, and detector 5 element 19-56 is removed. Movement of element 19-56 may be accomplished by robotics, or alternatively, detector element 19-56 may be handled manually. If detector element, 19-56 is film, it may be moved to an automatic developing machine such as an X-Omat before 10 being exposed to detector 1-40.

In the preferred embodiment of detector 1-40, each membrane unit 15-50 can be automatically extended out of membrane unit array 1-30 by a stepper motor or other linear driver to be provided to detector 15-40. 15 Detector 15-40, in turn, can move automatically between stations 15-32 along array 1-30 on rails 15-150, or other such tracks, between exposures. To further enhance the speed of detection, a plurality of detectors 15-40 may be used, enabling patterns on 20 different membranes 16-51 to be observed simultaneously.

Detector 1-40 and detector element 19-56 will, of course, be dependent on the method of labeling used by the oligomer synthesizer. For instance, if the 25 method of labeling employs radioactivity, detector element 19-56 may be a Beta-ray sheet, and detector 1-40 may be a scanning laser beam. If detector element 19-56 is film, detector 1-40 may be a CCD-based scanner.

30 Alternatively, a chemiluminescent labelled oligomer may be used for hybridization to sequencing patterns bound to membrane 16-51. After the appropriate chemical reactions (see for example, S. Beck et al., supra) light emitting patterns may be 35 read directly from membrane unit 16-50 into a CCD

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camera for detection, as shown in FIGS. 14 and 15. Regardless of the method of detection, a plurality of detectors 1-40 are preferably included in sequencer 1-1 to detect patterns from different membranes 16-51 5 simultaneously. Hybridized sequencing patterns detected by detector 1-40 are then transferred to computer 1-10, for instance by a series of electrical signals.

As with the other elements of sequencer 1-1, 10 the computer control of operations within detector 1-40 is ultimately controlled by computer 1-10, but an additional computer, microprocessor or microcontroller that is associated with detector 1-40 may control particular operations within detector 1-40.

15 Communication between detector 1-40 and computer 1-10, or between a separate computer device associated with detector 1-40 and computer 1-10, occurs over line 1-14. Such communication from computer 1-10 informs detector 1-40 of the membrane unit to be detected in conjunction 20 with informing array 1-30 of the membrane unit to make available for detection. Computer 1-10 is informed of the status of the detector, and is ultimately provided with electrical signals generated by detector 1-40 that represent the patterns of hybridized oligomers.

25 In the preferred embodiment, sequences are constructed in accordance with a walking algorithm by computer 1-10. Computer 1-10 may be provided with a two dimensional pixel image of the sequencing patterns on membrane 16-51 detected by detector 1-40. Such 30 images are then translated into a pattern of letters corresponding to the sequence represented by those images, thus forming sets of short DNA sequences. For example, computer 1-10 may use currently available computer software to translate the graphical images 35 observed by detector 1-40 into a series of

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corresponding letters A, C, G and T, such as:

"Replica" (G. Church, Harvard Medical School, Boston, MA.); BioImage (Ann Arbor, MI); or Protein and DNA Imagewear (Huntington Station, NY.). The sequence from

5 the sequencing lanes of each sample on membrane 16-51 is then combined with sequence obtained from other DNA samples on membrane 16-51 to construct a longer nucleic acid sequence.

Computer 1-10 assembles the sets of nucleic acid sequences based on regions of overlapping bases.

10 A region of overlap may contain for instance, 10 nucleotides, although it is more likely that overlaps of several hundred nucleotides will be generated. The sequence from each membrane 16-51 is then assembled

15 onto previously determined sequence stored in the memory of computer 1-10, again based on regions of overlapping bases. Commercially available software developed for shotgun sequence assembly may be used to combine such short nucleic acid sequences into a final

20 overlapped sequence. Examples of such packages are: GCG (University of Wisconsin); Eugenes (Baylor School of Medicine); and DNA Star (Madison, WI.).

Furthermore, the standard software assembly problems are avoided when utilizing the method and apparatus of

25 the present invention, because instead of requiring shotgun matching of random sequences, all that is required is the addition of new sequence in an orderly fashion to the end of previously defined sequence, a much simpler procedure. Because computer 1-10 stores

30 the sequence of the oligomers that were used to hybridize each membrane, assembly may be conducted in a straightforward fashion. Patterns from other membranes hybridized with other oligomers are used to assemble sequence for the opposite strand. If the assembled

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sequence does not overlap with previously determined sequence, it is stored separately.

For each next iteration of hybridization, computer 1-10 predicts the next set of oligomers based 5 on analysis of the most distant accurate unique sequences within the newly generated sequence. For instance, computer 1-10 may direct synthesis of oligomers complementary to the sequence lying within 20-40 bases from the end of the newly generated 10 sequence. Computer 1-10 directs synthesizer 1-20 to synthesize that next predicted set of oligomers, and selects a corresponding membrane 16-51 from array 1-30 for hybridization with each predicted oligomer.

Corresponding membrane 16-51 is selected based on the 15 nucleic acid sequencing patterns bound thereto and on the availability of membrane 16-51 at the time synthesis of the predicted oligomer is complete.

Control of sequencer 1-1 by computer 1-10 enhances the overall speed of sequencing by enabling 20 synthesizer 1-20 to produce sets of multiple oligomers continuously. Computer 1-10 can generally predict a next set of oligomers to be hybridized within some of membrane units 16-50 before completion of synthesis of the previously selected set of oligomers, which are to 25 be hybridized within other of membrane units 16-50.

Computer 1-10 accordingly directs synthesizer 1-20 to synthesize that next set of predicted oligomers as soon as synthesizer 1-20 completes synthesis of the previously predicted set of oligomers. Thus, 30 subsequent next sets of oligomers are continually synthesized. As a consequence of this interaction between computer 1-10 and synthesizer 1-20, next predicted oligomers are more likely to be available for hybridization within membrane units 16-50, thereby 35 reducing total sequencing time.

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To start the sequencing walks, a variety of starting sequences must be available in order to synthesize a set of oligomers that will be complementary to regions of the unknown sequence. Such 5 oligomers are referred to herein as "starting oligomers." When sequencing unknown genomic DNA directly, known sequences already contained within worldwide data banks may be utilized for this purpose. Additionally or alternatively, the DNA may be cleaved 10 with one or more enzymes recognizing only a small number of nucleotides and shotgun cloned. Short stretches of sequence from multiple clones may then be read using primers homologous to the vector sequences. Similarly, the sequence of starting oligomers for 15 cloned DNA may be determined by sequencing clones containing progressive deletions using primers homologous to vector sequences, such that a short stretch of sequence is read every 1-2 kb.

In the preferred embodiment, starting 20 oligomers of random sequence are synthesized. The length of such random starting oligomers is calculated based on the complexity of the DNA to be sequenced, such that each starting oligomer should hybridize to a DNA sequence occurring only once in the DNA. For 25 example, statistically, any given 11-mer should hybridize only once to DNA having a complexity of two megabases. The random oligomers are then used to probe Southern blots to determine if they identify unique sequences in the DNA. The computer program preferably 30 begins with a collection of approximately 500-1000 random oligomers which identify unique sequence for every megabase of DNA.

Before instructing synthesizer 1-20 to synthesize a predicted oligomer, computer 1-10 will 35 perform various tests on the sequence of that oligomer

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as a check. Among these tests will be a determination of the secondary structure formed by the sequence. Computer 1-10 will not direct the synthesis of any oligomer capable of forming a hairpin because oligomers 5 that form hairpins may be inefficiently synthesized and may hybridize inefficiently as well. Instead, computer 1-10 will read along the DNA sequence enough bases in the 5' or 3' direction to pick a new short stretch of sequence that will not include the hairpin region and 10 direct synthesis of a new oligomer based on that sequence.

Computer 1-10 will also determine if the next predicted oligomer has previously been used, or if the next predicted oligomer is located within a region in 15 which sequence has already been determined for several hundred bases in both directions. If either of these conditions is found, computer 1-10 does not direct synthesis of that oligomer, but instead directs synthesis of a new starting oligomer.

20 Computer 1-10 also determines if the newly generated sequence closes a region of DNA. A region of DNA may be closed either by walking inward from both directions, or by having one walk meet the beginning of an adjacent walk in the same direction. When the newly 25 generated sequence is found to close a region of DNA, computer 1-10 shifts its analysis to the most 5' and 3' ends of the region, and directs synthesis of oligomers according to that sequence. Computer 1-10 may also begin an independent determination of sequence in 30 another region, using new starting oligomers.

When the sequence of a given sample is completed, no new oligomers can be made that have not been previously made, or that do not lie within a region of known sequence. In other words, all walks 35 will have come to an end by overlapping other walks,

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and all starting oligomers will have been used. At this point, the computer will signal that analysis is complete.

Computer 1-10 continuously performs various tests on the newly constructed sequence as it performs the automated multiplex walking. After constructing the sequence derived from each membrane 16-51, the computer will first compare the two complementary DNA strands to search for a mismatch. The most likely cause of mismatches is compression of the pattern of DNA molecules corresponding to sequences containing stretches of C's or G's. This usually occurs because the DNA molecules fold back on themselves so that mobility during size separation does not correlate smoothly with length. Although compressions may occur on both strands, they usually do not occur opposite one another.

When a mismatch between the two strands is found, computer 1-10 may direct synthesis of new oligomers for each strand of DNA close to the trouble spot. These oligomers are then used to probe additional sets of membranes that contain different restriction enzyme digestion products of the same DNA.

Alternatively, computer 1-10 may direct synthesis of modified oligomers capable of hybridizing to sequence ladders generated from DNA that was previously treated with bisulfite. Such treatment causes cytosines in the DNA to be converted to uracils, thereby eliminating compressions. [See, e.g., N. Okada et al., J. Biochem., 91, pp. 1281-91 (1982).] If computer 1-10 cannot resolve a particular mismatch by either of these methods, it will flag the sequence output for later human inspection and intervention. Sequencer 1-1 may continue to operate, however, because

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other portions of the nucleic acid are still capable of being sequenced.

Certain sequence elements, such as middle and highly repetitive DNA, may not be amenable to sequence 5 analysis by automated sequencer 1-1. Computer 1-10 is able to identify a repeated sequence as a DNA element that has two or more regions apparently flanking it on the 5' and/or 3' sides. Computer 1-10 may not be able to sort the connection across the repeat, and in those 10 cases it will flag the sequence output for later human inspection and intervention.

The higher the complexity of the DNA being sequenced, the more likely it is that such elements will be present. For this reason, it may be 15 advantageous to clone large fragments of high complexity DNA into yeast artificial chromosomes or other vectors, so that repetitive sequences will occur with a decreased frequency. In addition, oligomers derived from multi-copy genes, or derived from 20 conserved portions of genes contained within gene families, may hybridize to multiple sequencing ladders simultaneously, and no discrete sequence will be discernable.

When any of the above situations arise, 25 computer 1-10 will flag the sequence output for later human inspection and intervention so that these regions may be sequenced by other means to determine the connectivity of the sequence across the region. For instance, oligomers derived from the closest unique 30 sequence may be used to screen libraries containing cloned DNA. Once a clone is obtained, it may be mapped and sequenced by methods well known to those skilled in the art, for instance, by Southern blotting and sequencing of progressive deletion mutants. 35 Alternatively, PCR could be used to generate sequence

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across the repeat. Meanwhile, computer 1-10 would direct the synthesis of a new starting oligomer in order to begin sequence analysis in a different region.

It is also conceivable, particularly when 5 sequencing highly complex genomic DNA directly, that there may exist regions of DNA in which there are no restriction sites. Such regions will not be amenable to sequencing more than several hundred bases from the closest restriction site on each side. In such a case, 10 the oligomer hybridization will not yield discernable sequencing patterns; this is diagnostic of the fact that these oligomers are derived from regions in which there are no restriction sites. Again, upon encountering such a region, computer 1-10 will flag the 15 sequence output for later human inspection and intervention so that this area may be sequenced by other means, for instance, by isolating the DNA with the aid of the diagnostic oligomer, fragmenting it by sonication or shearing, and then sequencing, either 20 before or after cloning.

Thus, it will be apparent that the combination of the four principal components of sequencer 1-1 into a unified apparatus creates a fully automated sequencing machine that rapidly determines 25 the sequence of a nucleic acid molecule by synthesizing multiple oligomers simultaneously, hybridizing oligomers to multiple membranes 51 simultaneously, analyzing the sequence and predicting next sets of oligomers.

30 It will be apparent to those skilled in the art that the invention described herein can be practiced by other than the embodiments disclosed herein, which are presented for the purposes of illustration and not limitation, and the present 35 invention is limited only by the claims that follow.

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A decorative border consisting of a repeating pattern of black asterisks (\*). The border is approximately 100 pixels wide and 10 pixels high, surrounding the main content area.

## Program SOLARSYNTH

## APPENDIX A

## Solenoid Array Synthesizer.

Print\*, ' Copyright (C) 1990'  
Print\*, ' President and Fellows of Harvard College'  
Print\*, ' Steve Kieffer-Higgins and George M. Church'  
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Currently set up for standard CE-phosphoramidite DNA synthesis and for Omega 992/915 RS232 interface from Omega Engineering.

To set up vax: set term/perm/eight/speed=9600 txa4  
To set up Omega: connect 50-wire cable from multiplexer  
to relay block. Connect 5 VDC and 12 VDC supplies to  
relay block. Connect the 25-pin control cables to the  
appropriate solenoid and valve arrays.

Test with TRS-100 TELCOM stat=8711E : 9600 baud, 7 bits, ignore parity, 1 stop bit, line enable

### Modification History:

Adapted from Valve.for 2-mar-87 to SOL4S on 9-Sep-89  
by George Church

to SIAS\_CPHOS\_1 and OMEGA\_CPHOS\_1 on 25-jun-90  
by Steve Kieffer-Higgins

to SOLARSYNTH on 28-Jan-91 by Steve Kieffer-Higgins

Current setup piston-col#1 on far right

Plate-col . . . 12 11 10 9 8 7 6 5 4 3 2 1  
 Plate-col . . . Ox - Cp - T - - - A G C T

Piston  
col  
2 1

Piston	8	16	8	—	
row	7	15	7	—	
	6	14	6	—	
	5	13	5	—	solenoid control numbers
	4	12	4	—	
	3	11	3	—	
	2	10	2	—	
	1	9	1	—	

Lower solenoid tier: 1,3,5,7,10,12,14,16  
Upper solenoid tier: 2,4,6,8,9,11,13,15

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For this test program only the lower tier is in use:  
1,3,5,10,12,14

Valve block: Upper row, with switches to the left, are #'s 1-12, and the lower row is numbered 13-24. In this run valves 7 & 8 are not used. The monomer and tetrazole valves are currently sharing relays.

Omega relay: This run uses only one relay block consisting of 32 switches, which is currently the limiting factor for capping as well as the total possible oligos which can be made in a single run. These are the current requirements for relays assuming a two-part capping reaction:

**MONOMERS: 12 RELAYS and 16 VALVES**

Monomers require 1 relay to simultaneously switch of 2 valves, monomer and tetrazole. Each also requires a dedicated rinse and vacuum valves. Thus, each monomer requires 4 valves controlled by 3 relays for a total of 16 valves and 12 relays.

**OXIDATION: 3 RELAYS and 3 VALVES**

The oxidizer is a one part reagent which requires dedicated rinse and vacuum valves.

**CAPPING: 3 RELAYS and 4 VALVES**

The capping reagent is a two-part mixture which requires dedicated rinse and vacuum valves.

**TCA: 3 RELAYS and 3 VALVES**

TCA is a one part reagent which requires dedicated rinse and vacuum valves.

**MOTOR: 4 RELAYS**

The motor requires 2 relays at 5VDC. Since the relay blocks can switch different voltages in groups of 4 and the valves all require 12VDC, the motor effectively takes 4 relays out of the pool.

**TOTAL: 29 RELAYS and 26 VALVES for reagent delivery and trough movement.**

**SOLENOIDS: 2 RELAYS EACH**

The solenoids require 12VDC to pull up a tip and switching to 5VDC to maintain a hold on the tip. The solenoids reach a temperature greater than 100C when in an array with continuous application of 12VDC, but reach about 35C at 5VDC. Thus each Omega OM915 relay module can control 16 individual solenoids, or a total of 6 OM915 units for 96 solenoids.

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The present set up with a single OM915 allows the control of 6 solenoids if we work without a capping reaction. Not capping saves 3 relays and 4 valves. Based on the architecture of the relay blocks, which allows voltages in groups of 4 to be switched, we would be reduced to only 3 different oligos per run were we to incorporate a capping reaction.

**RELAY/VALVE ASSIGNMENTS:**

OMEGA RELAY	REAGENT	VALVE
1	tBHP	19
2	TCA	20
3	A monomer	21
	TET-A	9
4	C monomer	22
	TET-C	10
5	G monomer	23
	TET-G	11
6	T monomer	24
	TET-T	12
7	vac-tBHP	1
8	vac-TCA	2
9	vac-A	3
10	vac-C	4
11	vac-G	5
12	vac-T	6
13	CH3CN-tBHP	13
14	CH3CN-TCA	14
15	CH3CN-A	15
16	CH3CN-C	16
17	CH3CN-G	17
18	CH3CN-T	18
25	Motor - Drive	25
26	Motor - CW/CCW	26
19-24	12V	solenoids
27-32	5V	solenoids

Each step entails a CH3CN rinse that rinses both the reagent troughs and the tips used in that trough during that step. In this way no additional valves/relays are required to effect a rinse. Additionally, the same control loop can be used for both the chemical and rinse steps with only an update in the control variables. This feature will be most useful in preventing cross-contamination of adjacent tips in the first rinses after monomer addition.

Manual synthesis cycle as performed SKH 14-jun-90

Pos	
1.	CH3CN 20"
2.	TCA 5 x 10"
	CH3CN 20"

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3. Base	180"
CH3CN	20"
4. Cap	120"
CH3CN	20"
5. Oxid	60"
CH3CN	20"
 integer*4	lenmax ! maximum length of oligos to be made
 integer*4	colmax ! maximum number of columns - hardware dependant
 integer*4	rowmax ! maximum number of row - hardware dependant
 integer*4	stepmax ! number of synthetic steps + 1 (initiation)
 integer*4	troughmax ! number of reagent troughs, currently 6 BUT must be incremented to 7 when capping is added.
 integer*4	trough_unit ! the number of motor steps to move 9mm
 integer*4	rxnstep ! the number of substeps per synthetic step. Currently 3: the actual reaction, vacuuming out the trough, and adding rinse.
 integer*4	v1vmax! The number of commands that must be sent to a given trough, currently 6: open/close reagent, vacuum and rinse.
 parameter(	lenmax=38, colmax=2, rowmax=3, stepmax=5
parameter(	troughmax=6, trough_unit=33, rxnstep=3,
v1vmax=6	
 real*4	pos(stepmax) !The absolute position of a reagent trough within the trough plate - defined by user in SOLARSYNTH.TMR
 real*4	pumptm(stepmax,rxnstep)!The length of time to hold a valve open - defined by user in SOLARSYNTH.TMR
 real*4	wait(stepmax)!Reaction time per step - defined by user in SOLARSYNTH.TMR
 real*4	wait2(stepmax)!Rinse time per step - defined by user in SOLARSYNTH.TMR

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logical	long !toggles between full length reaction steps. When TRUE the times defined in SOLARSYNTH.TMR are converted from minutes to seconds; otherwise the minutes are treated as seconds.
logical	yescheck ! required by GETSTR
integer*4	ldrvcmand, lcmmnd, lreset, lcpureset ! Length of command strings sent to WRIBOT
integer*4	step ! Control variable which keeps track of where in the synthesis cycle the program is.
integer*4	loopbeg, loopend ! the bounds in which STEP operates
integer*4	mono ! the number of monomers, currently 4
integer*4	NC, NR ! the actual number of columns and rows of oligos to be made which will vary with each run
integer*4	len ! the length of the longest oligo to be made per run
integer*4	cyc ! control variable which operates within the bounds of LEN, above
integer*4	ld(colmax, rowmax), lu(colmax, rowmax) ! length of commands that raise (lu) and lower (ld) the solenoids, required by WRIBOT
integer*4	seqm(colmax, rowmax, lenmax) !integer representation of the oligos to be made, where A=1, C=2 etc
character*1	mlis(20) ! reference against which an element of SEQM above is compared when the program is testing to see if a solenoid is to drop its tip. When the number in the SEQM array matches the subscript of MLIS, the tip is dropped.
character*30	Chem(stepmax), chem2(stepmax) !Description of each step: Chem=rxn, chem2=rinse
character*38	seq(colmax, rowmax)

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```

character*60  drive(3) !Holds the commands that drive
              the motor: 1=pulse, 2=CW, 3=CCW

character*60  check !dummy var required for GETSTR

character*60  rxnvlv(troughmax, vlvmax) !holds the
              open and close commands for the valves

character*60  cpureset,reset !Reset commands for Omega
              CPU and OM915

character*60  down(colmax,rowmax), up(colmax,rowmax) !
              Lift and drop commands for the solenoids
*****  

MAINLINE

call INITIALIZE( chem, chem2, colmax,
2           cpureset, down, drive, lcpureset, ld,
ldrvcmmnd,
3           len, lenmax, long, loopbeg, loopend, lreset,
lcmmnd,
4           lu, mlis, mono, pumptm, rxnstep,
5           NC, NR, pos, reset, rowmax, rxnvlv, seq,
seqm,
6           stepmax, trough_unit, troughmax, up, vlvmax,
wait, wait2)

call getstr(check,'Hit RETURN to begin
synthesis,', '<CR>', yescheck)

Do cyc=2,Len
  Do step = loopbeg, loopend

    if (step.eq.2) then
      call ADD_MONOMER (chem, chem2,
2
colmax,cyc,down,drive,ld,lenmax,ldrvcmmnd,lcmmnd,
3   lu, mlis, mono, NC, MR, pos, pumptm, rxnstep,
4   rowmax, rxnvlv, seqm, step, stepmax,
5   trough_unit, troughmax, up, vlvmax, wait, wait2)

    else
      call NON_ADDITIVE-CHEMISTRY(chem, chem2,
2
colmax, down, drive, long, ld,
3   ldrvcmmnd, lcmmnd, lu, rxnstep,
4   NC, pos, pumptm, rowmax, rxnvlv, step,
5   stepmax,troughmax,trough_unit, up, vlvmax, wait,
wait2)

    end if
End do ! step

```

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```
End do ! cyc
call wribot( reset(1:lreset) )
END !solarsynth

*****
subroutine NON_ADDITIVE_CHEMISTRY(chem, chem2,
2      colmax, down, drive, long, ld,
3      ldrvcmmd, lcmmnd, lu, rxnstep,
4      NC, pos, pumptm, rowmax, rxnvlv, step,
5      stepmax, troughmax, trough_unit, up, vlvmax,
wait,
      wait2)

coordinates TCA, oxidation and capping steps

integer*4
      colmax, rowmax, stepmax, troughmax, rxnstep, vlvma
      x

real      time, delta, oldpos
real*4    pos(stepmax), pumptm(stepmax, rxnstep)
real*4    wait(stepmax), wait2(stepmax)
logical   long
integer*4  NC, vlv, i, step, trough_unit
integer*4  ldrvcmmd, lcmmnd
integer*4  ld(colmax, rowmax), lu(colmax, rowmax)
character*30 Chem(stepmax), chem2(stepmax)
character*60 drive(3)
character*60 down(colmax, rowmax), up(colmax, rowmax)
character*60 rxnvlv(troughmax, vlvmax)

if (step.eq.3) return !cap
if (step.eq.4) then
      vlv=1 !oxidize
      oldpos=5
end if
if (step.eq.5) then
      vlv=2 !TCA
      oldpos=12
end if

call MOVEX (oldpos, pos(step), drive, ldrvcmmd,
2                      lcmmnd, trough_unit)

fill trough: reagent valve = 1
call
pump(rxnvlv(vlv,1), rxnvlv(vlv,2), lcmmnd, pumptm(step,1))

react tips:
```

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```

print*,chem(step)
write(66,'(a)')chem(step)
delta=0
time=secnds(0.0)
print*, 'REACTION TIME=',(wait(step)/60),'minutes'
write(66,'(a,f,a)')'REACTION
TIME=',(wait(step)/60),'minutes'
do while(delta.lt.wait(step))
    delta=secnds(time)
    call dip_all_cols(colmax, down, drive, ld,
1drvcmmd,lcmmnd,
2      lu, NC, pos, rowmax, step, stepmax, trough_unit,
up)
end do! while

rinse trough

print*,chem2(step)
write(66,'(a)')chem2(step)
delta=0
time=secnds(0.0)
print*, 'RINSE TIME=',(wait2(step)/60),'minutes'
write(66,'(a,f,a)')'RINSE
TIME=',(wait2(step)/60),'minutes'
call pump(rxnv1v(vlv,3), rxnv1v(vlv,4), lcmmnd,
pumptm(step,3))
do while (delta.lt.wait2(step))

remove reagent:

rinse:
print*,chem2(step)
write(66,'(a)')chem2(step)
call pump(rxnv1v(vlv,5), rxnv1v(vlv,6), lcmmnd,
pumptm(step,2))

do i = 1, 5
    call dip_all_cols(colmax, down, drive, ld,
1drvcmmd, lcmmnd,
2      lu, NC, pos, rowmax, step, stepmax,
trough_unit, up)

end do !dip
call pump(rxnv1v(vlv,3), rxnv1v(vlv,4), lcmmnd,
pumptm(step,3))
delta=secnds(time)

end do !while

return

end ! non-additive-chemistry

```

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```
*****
subroutine INITIALIZE( chem, chem2, colmax,
2      cpureset, down, drive, lcpureset, ld,
ldrvcmmnd,
3      len, lenmax, long, loopbeg, loopend, lreset,
lcmmnd,
4      lu, mlis, mono, pumptm, rxnstep,
5      NC, NR, pos, reset, rowmax, rxnvlv, seq, seqm,
6      stepmax, trough_unit, troughmax, up, vlvmax,
wait, wait2)

integer*4      col, row, colmax, rowmax, stepmax, rnxstep, vl
vmax
real*4         pos(stepmax), pumptm(stepmax, rxnstep)
real*4         wait(stepmax), wait2(stepmax)
logical        long, yescheck
integer*4      troughmax, loopbeg, loopend, stepnum
integer*4      i, b, bb, q, p, m, mono, trough_unit
integer*4      Len, lenmax, seqm(colmax, rowmax, lenmax)
integer*4      NC, NR, vlv, ldrvcmmd, lcmmnd, prm
integer*4      ld(colmax, rowmax), lu(colmax, rowmax)
integer*4      lreset, lcpureset, rotn
character*1    mlis(20), newdir, oldir
character*8    waitasec
character*30   Chem(stepmax), chem2(stepmax)
character*38   seq(colmax, rowmax)
character*60   dummyline
character*60   cpureset, reset, halt, CW, CCW
character*60   down(colmax, rowmax), up(colmax, rowmax)
character*60   drive(3), str, check, prmvlv, vacprm
character*60   rxnvlv(troughmax, vlvmax)
```

Colmax & rowmax determine the number of pins used in the program. With only one OM-915 we have room for only 6 individually addressable solenoids, 2 columns x 3 rows. Stepmax = 6, which reflects the initial step of starting the synthesis and 4 synthetic reactions: 2=monomer addition, 3=cap (not used in this version), 4=oxidation, 5=trityl release. Each step has a CH3CN rinse at the end.

Loopbeg= 2 ! Start with preloaded detritylated solid phase  
 Loopend= 5 ! 4 actual synthetic steps, if we use capping

Solenoid columns begin with one on the left.  
 At X=0.0, pos=1 lines up with col=1, pos=2 with col=2 etc.

mono = 4 ! number of monomer solutions 4 = ACGT

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```
call getstr(str,' Long(full) version or rapid
check','rapid', long)
long=.not.long

mlis(1)='A'
mlis(2)='C'
mlis(3)='G'
mlis(4)='T'

Chem( 1)= 'M  start'
pos( 1)= 8 ! trough is moved to monomer pos from TCA
pos
```

The following routines establish the timing parameters for each step. The user may input any timing parameter as minutes in real numbers, ie 3.5 instead of 3:30. Input file follows this format:

```
filename: SOLARSYNTH.TMR

line#      content
1.        MINUTES[TAB]TROUGH POS[TAB]TROUGH FILLING
TIME

2.        MONOMER COUPLING
3.        rxn time [spaces] trough pos [spaces] time to
fill trough
4.        MONOMER RINSE
5.        rinse time [spaces] time to fill trough
6.        MONOMER TROUGH VACUUM
7.        time to empty trough

8.        CAPPING REACTION
9.        rxn time [spaces] trough pos [spaces] time to
fill trough
10.       CAPPING RINSE
11.       rinse time [spaces] time to fill trough
12.       CAPPING TROUGH VACUUM
13.       time to empty trough

14.       OXIDATION
15.       rxn time [spaces] trough pos [spaces] time to
fill trough
16.       OXIDATION RINSE
17.       rinse time [spaces] time to fill trough
18.       OXIDATION TROUGH VACUUM
19.       time to empty trough

20.       TCA TRITYL RELEASE
21.       rxn time [spaces] trough pos [spaces] time to
fill trough
22.       TCA RINSE
23.       rinse time [spaces] time to fill trough
```

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24. TCA TROUGH VACUUM  
 25. time to empty trough

```

print*, 'Opening SOLARSYNTH.TMR for timing
parameters...'
open(11, name='solarsynth.tmr', status='old', readonly)

take care of header:
read(11, '(a)') dummyline
read the 4 sets of timing parameters:
do i= 2, 5
  read(11, '(a)') chem(i)
  write(66,*) 'chem(i)', chem(i)
  read(11,*) wait(i), pos(i), pumptm(i,1)
  write(66,*) 'wait(i), pos(i), pumptm(i,1)', wait(i), pos(i),
  pumptm(i,1)
  read(11, '(a)') chem2(i)
  write(66,*) 'chem2(i)', chem2(i)
  read(11,*) wait2(i), pumptm(i,2)
  write(66,*) 'wait2(i), pumptm(i,2)', wait2(i), pumptm(i,2)
  read(11, '(a)') dummyline
  read(11,*) pumptm(i,3)
  write(66,*) 'pumptm(i,3)', pumptm(i,3)
end do !i
if (long) then
  do i=2,5
    wait(i)=wait(i)*60
    wait2(i)=wait2(i)*60
  end do
end if

read sequence data:
print*, 'Opening SEQUENCE.IN for synthesis
parameters...'
Open(10, name='sequence.in', status='old', readonly)
Len=0
Do col=1, colmax
  Do row = 1, rowmax
    read(10, '(a)', err=9, end=9) seq(col, row)
    determine Len, flip 3' to 5', and translate to
    monomer #
    do b=1, lenmax
      seqm(col, row, b)=0 ! initialize to no monomer
coupling
    end do
    bb=0
    do b=lenmax, 1, -1
      do m=1, mono
        Check Upper and lowercase ACGT, etc.:
        If( char(ichar(seq(col, row)(b:b))-32).eq.mlis(m)
        &           .or. seq(col, row)(b:b).eq.mlis(m) ) then

```

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```

bb=bb+1
if(bb.eq.1) write(66,*) ' '
seqm(col,row,bb)=m
writes a #:1,2,3 or 4, corresponding to
a monomer
&
write(66,'(a,4i4,x,a)')' Col,Row,base,m'
, col, row, bb, m, seqm(col, row) (b:b)
goto 1

End If
End do ! m
End do ! b
If( Len.lt.bb) Len=bb
End do ! row
End do ! col
col=colmax ! on leave fortran loop index goes +1 beyond
limit!
continue

```

interface resets:

```

write(cpureset,'(a)')'@RESET'//char(13)//char(10)
write(reset,'(a)')'#1 RESET'//char(13)//char(10)
lcpureset=8
ltrdry=10

```

valve assignments:

We are currently set up for 6 reagent troughs, each with dedicated reagent, vacuum & rinse valves. Capping is not used. If we add capping later the omega relay block will have to be rewired to reflect this and the control var TROUGHMAX should be increased to 7. The variable q reflects the actual Omega relay number. Once capping has been integrated into the program the wiring harness must be rewired so that relay 1=tBHP, 2=CAP, 3=TCA, 4=A monomer, etc.

```

q=1
do p-1, vlvmax,2
    do i=1, troughmax
        write(rxnvvlv(i,p),'(a,i2,a)')'#1,SW',q,'=1'
2           //char(13)//char(10)
        write(rxnvvlv(i,p=1),'(a,i2,a)')'#1,SW',q,'=0'
2           //char(13)//char(10)
        q=q+1
    end do ! vlvmax
end do ! troughmax

```

relays 19 - 24 = solenoid 12V

```

MOTOR - drive req 5V
write(drive(1),'(a)')'#1,SW25=1;SW25=0'//char(13)//char
(10)

```

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```

MOTOR - CW/CCW
write( drive(2) ,'(a)''#1,SW26=1'//char(13)//char(10)
write( drive(3),'(a)''#1,SW26-0'//char(13)//char(10)

ldrvcmmnd = 19
lcmmnd = 11

relays 27-32 = solenoid 5V

lift & drop commands for the solenoids:

m=18 !1st 12V solenoid assignment on OM-915 is next, 19
Do col=1,colmax ! col = x
    Do row=1,rowmax ! row = y
        ld(col,row)=11 ! 11 characters long
        lu(col,row)=25 ! 25      "      "
        m=m+1
        write(down(col,row),'(a,i2,a)''#1,SW',m+8,'=0'
2 //char(13)//char(10)
        print*, 'COL, ROW, DOWN(col,row)', col ,row,
down(col,row)
        write( up(col,row),'(a,i2,a,i2,a,i2,a)')
2 '#1,SW',m,'',m+8,'=1'//char(13)//char(10)//
3 '#1,SW',m,'=0'//char(13)//char(10)
        print*, 'COL, ROW, UP(col,row)', col ,row, up(col,row)
        End do

    End do

    print*, 'CPU RESET'
    write(66,'(a)')'CPU RESET'
    call wrrobot(cpureset (1:lcpureset))

    print*, 'MULTIPLEXER RESET'
    write(66,'(a)')'MULTIPLEXER RESET'
    call wrrobot(reset (1:lreset))

NC=col-1
MR=row-1
If(NR.le.0) then
    NR=rowmax
    NC=NC-1
End if

Initialize all relays to the no power state, then set
to "UP".
Do col = 1,NC
    Do row = 1,rowmax
        If(col.eq.NC.and.row.gt.NR) goto 2
        call wrrobot( up(col,row)(1:lu(col,row)) )
    End do ! row
End do ! col

```

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```

continue
print*, 'Enter the number which corresponds to desired
operation:'
print*, ''
print*, '1. Position trough unit.'
print*, '2. Prime reagents.'
print*, '3. Activate solenoids.'
print*, '4. Initiate synthesis.'
print*, ''
call getint(prm,'Your choice?',999)
if (prm.eq.999) goto 2
if (prm.lt.1) goto 2
if (prm.gt.4) goto 2

if (prm.eq.1) call
position_trough(drive,lcmmnd,ldrvcmmd,
2           trough_unit)

if (prm.eq.2) call
prime_reagents(cpureset,lcmmnd,lcpureset,
2           lreset,reset)

if (prm.eq.3) call
solenoids(colmax,down,ld,lu,NC,rowmax,up)

if (prm.eq.4) goto 3

goto 2

continue

return

end !initialize

*****
subroutine DIP_ALL_COLS (colmax, down, drive, ld,
ldrvcmmd, lcmmnd,
2           lu, NC, pos, rowmax, step, stepmax,
trough_unit, up )

Dips all tips one column at a time into a given trough

integer*4      col, row, rowmax, stepmax, colmax
real*4         pos(stepmax), newpos
integer*4      NC, NR, step, trough_unit, ldrvcmmd,
lcmmnd
integer*4      ld(colmax,rowmax), lu(colmax,rowmax),
i,q
character*8     waitasec

```

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```

character*60    down(colmax,rowmax), up(colmax,rowmax),
                drive(3)

waitasec='0:00.50'
Do col = 1,NC
  do i=1, 5
    Do row = 1,rowmax
      If(col.eq.NC+1.and.row.gt.NR) goto 4
      Call wribot(down(col,row)(1:ld(col,row)) )
    end do ! row

  call waiter(waitasec)

  do q=1,5
    Do row = 1,rowmax
      Call wribot(up(col,row)(1:lu(col,row)) )
    end do ! row
  end do !q
  newpos=pos(step)-col
  end do ! i

  if (col.lt.NC) call movex
2  (pos(step)-
col+1,newpos,drive,ldrvcmmd,lcmmnd,trough_unit
  if (col.eq.NC) call movex
2  (pos(step)-
col+1,pos(step),drive,ldrvcmmd,lcmmnd,trough_unit
End do ! col
continue
return

end ! dip-all-cols

*****
subroutine ADD_MONOMER (chem, chem2,
2   colmax,cyc,down,drive,ld,lenmax,ldrvcmmd,lcmmnd,
3   lu, mlis, mono, NC, NR, pos, pumptm, rxnstep,
4   rowmax, rxnvlv, seqm, step, stepmax,
5   trough_unit, troughmax, up, vlvmax, wait, wait2)

Coordinates monomer addition

integer*4    colmax, rowmax, stepmax, lenmax, rxnstep, vlv
max
real        delta, time
real*4      pos(stepmax), pumptm(stepmax,rxnstep)
real*4      wait(stepmax), wait2(stepmax)
logical     long
integer*4    ldrvcmmd, lcmmnd, step, mono, cyc, q
integer*4    NC, NR, co, i, troughmax, trough_unit
integer*4    ld(colmax,rowmax), lu(colmax,rowmax)

```

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```

integer*4      seqm(colmax,rowmax,lenmax),firstmono,las
               tmono
character*1    mlis(20)
character*30   chem(stepmax), chem2(stepmax)
character*60   drive(3)
character*60   rxnvlv(troughmax, vlvmax)
character*60   down(colmax,rowmax), up(colmax,rowmax)

pump monomers:
firstmono = 3 !INCREASE TO 4 WITH CAPPING
lastmono = 6 !INCREASE TO 7 WITH CAPPING

do i=firstmono, lastmono
  call pump(rxnvlv(i,1), rxnvlv(i,2),
  lcmmnd,pumptm(step,1))
end do

while there is time: wait

call movex(pos(step-
1),pos(step),drive,ldrvcmmnd,lcmmnd, trough_unit)
print*,chem(step)
write(66,*)chem(step)
delta=0
time=secnds(0.0)
do while (delta.le.wait(step))
  call addition (cyc, colmax, down, drive,
  ld,ldrvcmmnd,lcmmnd,
  2  lenmax,lu,mlis,mono, NC, NR, pos, rowmax, seqm,
  step,
  3  stepmax, troughmax, trough_unit, up)

  delta=secnds(time)
end do! addition

while there is time : wait2
print*,chem2(step)
write(66,*)(chem2(step)
delta=0
time=secnds(0.0)

remove reagent:
do i= firstmono, lastmono
  call pump(rxnvlv(i,3), rxnvlv(i,4), lcmmnd,
  pumptm(step,3))
end do

do while (delta.le.wait2(step))
pump rinse:
  do i= firstmono, lastmono
    call pump(rxnvlv(i,5), rxnvlv(i,6), lcmmnd,
  pumptm(step,2))
  end do

```

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```

      do q=1, 5
         call addition
      (cyc,colmax,down,drive,ld,ldrvcmmnd,lcmmnd,
2         lenmax,lu,mlis,mono, NC, NR, pos, rowmax, seqm,
      step,
3         stepmax, troughmax, trough_unit, up)
      end do ! q
      delta=secnds(time)
      do i= firstmono, lastmono
         call pump(rxnv lv(i,3), rxnv lv(i,4), lcmmnd,
      pumptm(step,3))
      end do

      end do! rinse

      return

      end ! add monomer

*****  

subroutine ADDITION (cyc,colmax,down,
drive,ld,ldrvcmmnd,lcmmnd,
2   lenmax,lu,mlis,mono,NC, NR, pos,
rowmax,seqm,step,stepmax,
3   troughmax, trough_unit, up)

decides which tips drop into which monomer trough

integer*4      col, row, colmax, rowmax, stepmax,
lenmax
real*4         pos(stepmax), lastep, firstep
logical        long, drop
integer*4      b, bb, cyc, dip, loopbeg, loopend,
m,mono,step
integer*4      NC, NR, co, i, c, r, trough_unit,
troughmax,q
integer*4      ld(colmax,rowmax), lu(colmax,rowmax),
lcmmnd
integer*4      seqm(colmax,rowmax,lenmax),ldrvcmmnd
character*1    mlis(20)
character*8    waitasec
character*60   down(colmax,rowmax), up(colmax,rowmax),
drive(3)

waitasec='00:01.00'
firstep = pos(step)
lastep = pos(step)
drop=.false. !conditional for monomer dip
NC holds same value    COLMAX:
Do col = 1, NC+3

```

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```

    call MOVEX( lastep, firstep-col+1,
drive,ldrvcmmnd,
2           lcmmnd, trough_unit)

    pos(step) for monomers= -3, such that the
    first column of tips stands directly above
    the 'A' trough.
    When col=2, move is to position -2, etc.
    Do row = 1, rowmax
        bailout conditional:
    If(col.eq.NC+3.and.row.gt.NR) goto 7
        then the last column/row is past the end

    min(mono,col) returns the smallest #, mono,
which = 4,
        or col, which increments by 1 from 1 to NC+3
        first pas: m=1; loop is used once:only one
column
        could be over the first monomer trough.
        Second pass, m=2; loop used twice, 2 columns
are
        over monomer troughs:
    Do m=min(mono,col),1,-1
        co=col-m+1           ! first pass: co=1-1+1 =
1
        if(co.gt.NC) goto 11
        if(co.eq.NC.and.row.gt.NR) goto 11
!bailout conditional

    test to see if the integer held in
seqm(co.row.cyc),
        which corresponds to a monomer, is the same
as m
        First pass: m=1

    If(seqm(co,row,cyc).eq.m) then
        if it does write to output file & drop that
tip:
        write(66,'(a,4i3,x,a)')' Cyc,col,row,co,
base',
2           cyc,col,row,co, mlis(m)
           call wribot( down(co,row)(1:ld(co,row)))
)
        end if !
    end do !m
    continue
end do ! row
call waiter(waitasec)

    lift tips:cmmnd to all
do c=1,NC
    do r=1, rowmax
        if (c.eq.NC.and.r.gt.NR) goto 14

```

## SUBSTITUTE SHEET

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```

        do q=1,5
          call wribot( up(c,r)(1:lu(c,r)) )
        end do ! q
      end do !row
      end do ! col

      continue
      lastep=firstep-col+1
      drop=.false.

      End do ! col
      Continue
      call MOVEEx(lastep-
      1,firstep,drive,ldrvcmmnd,lcmmnd,trough_unit)

      return

      End ! addition

*****
subroutine wribot(data)

To output strings of unknown length to serial RS23 port
without terminal <CR> or other characters.
Version 1.0    Apr 2 1987    GMC

integer*2      ttchan,ttinit,iosb(4)
integer*4      i,SYS$QIOW,icode,funcw,efn
integer*4      LIB$GET_EF,LIB$FREE_EF
character*(*)  data

include '($iodef)'
external IO$_WRITEVBLK,IO$M_NOFORMAT

ttchan=ttinit()
funcw=ior(%loc(IO$_WRITEVBLK),%loc(IO$M_NOFORMAT))
icode=LIB$GET_EF(efn)
if (.not.icode) call LIB$SIGNAL(%VAL(icode))
icode = SYS$QIOW ( %val(efn),%val(ttchan),%val(
2 funcw),iosb,,,%ref(data),%val(len(data)),,%val(0),, )
if (.not.icode) call LIB$SIGNAL(%VAL(icode))
icode=LIB$FREE_EF(efn)
if (.not.icode) call LIB$SIGNAL(%VAL(icode))

return
end

*****
integer*2 function TTINIT()

```

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```

returns communication channel number for terminal-like
IO
for example through RS232C ports to the arm and stage.

logical      init,yestemp
integer*2    ttchan
integer*4    i,SYS$ASSIGN,icode
character*60  port

data init /.true./
This will keep these and only these values
save init,ttchan
around for use each time this routine is called

if(init) then
  port='txa4:'
  call getstr(port,' RS232 port 0-3 ',port,yestemp)
  icode = SYS$ASSIGN(port(:5), ttchan,, )
  If (.not.icode) call LIB$SIGNAL(%VAL(icode))
  init=.false.
end If
TTINIT = ttchan
return
end
*****
subroutine timer_sub()
include '($syssrvnam)'
integer*4 status
status=sys$wake(,)
return
end

*****
subroutine waiter (waist)
character*5 waist
include '($syssrvnam)'
integer*4 interval(2),status

external timer_sub
print*, ' ...waiting ',waist
status+sys$bintim('0 ://waist, interval)
print*, 'status=',status
if(.NOT. status) call lib$signal(%val(status))
status=sys$  setimer(, interval ,timer_sub,)
if(.NOT. status) call lib$signal(%val(status))
status sys$hiber()
if(.NOT. status) call lib$signal(%val(status))
return
end

*****
Subroutine MOVEX(oldpos,newpos,drive,ldrvcmmnd,
2           lcmmnd,trough_unit

```

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Sends a series of pulses to the OM915 to increment stepper motor. it decides which direction to turn the motor by comparing the old position to new position.

```
real      oldpos, newpos
real      pos, time
logical   yes, long
integer*4 lcmmnd, trough_unit, q, i, ldrvcmmd,
delta
character*8 waitasec
character*60 str
character*60 drive(3)
```

```
drive: 1=pulse on/off, 2=CW, 3=CCW
delta = int(oldpos-newpos)
```

```
if delta is positive then rotate CCW, if negative then
rotate CW
if (delta.ge.0) call wribot(drive(3) (1: lcmmnd))
if (delta.lt.0) then
    call wribot(drive(2) (1: lcmmnd))
    delta=delta*(-1)
end if!delta.lt.0
if (delta.eq.0) delta=1

waitasec='0:00.01'
do i + delta, 1, -1
    do q=1, trough_unit
        call wribot(drive(1) (1:ldrvcmmd))
        call waiter(waitasec)
    end do !q
end do !i

return
```

```
END !MOVE
```

```
*****
Subroutine Pump(vlvopn, vlvcls, lcmmnd, pumptm)
```

```
opens and closes a given valve for time value in PUMPTM
```

```
real      delta, time, pumptm
integer*4 lcmmnd
character*60 vlvopn, vlvcls

print*, 'pumptm=', pumptm
open valves
call wribot(vlvopn(1:lcmmnd))
wait for pumptm:
```

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```

delta=0
time=secnds(0.0)
do while(delta.lt.pumptm)
    delta=secnds(time)
end do! while

close valves
call wribot(vlvcls (1:lcmmnd))

Return

End !pump

*****
subroutine POSITION_TROUGH(drive, lcmmnd, ldrvcmmd,
2                      trough_unit

initializes trough to TCA position and allows user to
move trough
to any of the active trough positions

real*4          oldpos, newpos
logical         long, check, yescheck
integer*4        lcmmnd, ldrvcmmd, trough_unit, i
character*8      waitasec
character*60     drive(3), resp

position troughs

call getstr(resp,'Initialize the trough?
(Y/N)', 'Y', long)
if ((resp.eq.'N').or.(resp.eq.'n')) goto 97
if ((resp.eq.'Y').or.(resp.eq.'y')) goto 95
goto 96

print*, 'Position troughs so that column 1 is over the
TCA trough:'
print*, ' '
print*, '1. Turn off power to the stepper motor.'
print*, '2. Push trough unit ALL THE WAY to the left
(motor end).'
print*, '3. Turn on power to the stepper motor.
print*, ' '
print*, 'NEVER MANUALLY MOVE THE MOTOR WITH POWER ON!'
print*, ' '
print*, 'Make sure that your fingers are out of the
way!'

call getstr(check, 'Press RETURN when
done:', yescheck, long)
call wribot(drive(2) (1:lcmmnd))
waitasec='0:00.01'

```

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```
do i=1,365      !this puts trough 8 (TCA) under col
1
    call wribot(drive(1) (1:lcmmnd))
    call waiter(waitasec)
end do !i

oldpos=8

print*, ' '
print*, 'Enter the number which corresponds to
positioning'
print*, 'column 1 over the indicated trough:'
print*, ' '
print*, 'pos    col 2    col 1'
print*, '0.    %      -'
print*, '1.    G      T'
print*, '2.    C      G'
print*, '3.    A      C'
print*, '4.    -      '
print*, '8.    -      TCA'
print*, '9.    TCA    -'
print*, '10.   -      CAP'
print*, '11.   CAP    -'
print*, '12.   -      OX'
print*, '13.   OX    -'
print*, '14.   Reinitialize trough '
print*, '999.  Quit'
print*, ' '
print*, 'Unless 14 is entered the trough will
automatically'
print*, 'return to position 8 when you exit this
routine.'
print*, ' '

call getlreal(newpos,'Your choice?  Current
position:',oldpos)

if (newpos.eq.999) goto 98
if (newpos.lt.0) goto 97
if (newpos.eq.14) goto 96
if (newpos.gt.14) goto 97
if ((newpos.ge.5).and.(newpos.lt.8)) goto 97
if (newpos.eq.oldpos) goto 97
call movex(oldpos,newpos,drive,ldrvcmmnd,lcmmnd,trough-
unit)
oldpos=newpos
goto 97

continue
if (oldpos.ne.8) then
    newpos=8
    call
    movex(oldpos,newpos,drive,ldrvcmmnd,lcmmnd,
```

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```

2      trough_unit)
end if

return

end !position_trough

*****  

subroutine
PRIME_REAGENTS(cpureset,lcmmnd,lcpureset,lreset,reset)
gives user control over reagent valves

integer*4      prm, lcmmnd, lreset, lcpureset, i
logical        yescheck
character*8     waitasec
character*60    vacprm, prmvlv, cpureset, reset, check

print*, ' '
print*, "Vacuum valve will be open with all reagent
selections"
print*, ' '
print*, 'Enter the number corresponding to the valve:'
print*, '1. tBHP      7. tBHP vacuum    13. tBHP
rinse'
print*, '2. TCA      8. TCA vacuum     14. TCA
rinse'
print*, '3. A monomer  9. A vacuum      15. A
rinse'
print*, '4. C monomer  10. C vacuum     16. C
rinse'
print*, '5. G monomer  11. G vacuum     17. G
rinse'
print*, '6. T monomer  12. T vacuum     18. T
rinse'
print*, ' '
call getlint(prm,'Your choice? 999 to quit',999)

if (prm.eq.999) goto 88 !quit

if ((prm.gt.18).or.(prm.lt.1)) goto 89 !input out of
range

if (prm.lt.7) then !reagent valve
      write(vacprm,'(a,i2,a)') '#1,SW',prm+6,'=1'
2      //char(10)aacha 13)
      call wribot(vac,.m(1:lcmmnd))
      write(vacprm,'(a,i1,a)') '#1,SW',prm+6,'=0'
2      //char(10)//char(13)

end if

if (prm.gt.12) then !rinse valve

```

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```

        write(vacprm,'(a,12,a)')'#1,SW',prm-6,'=1'
2      //char(10)//char(13)
      call wribot(vacprm(1:lcmmnd))
      write(vacprm,'(a,82,a)')'#1,SW',prm-6,'=0'
2      //char(10)//char(13)
end if

if its anything else:
write(prmvlv,'(a,12,a)')'#1,SW',prm,'=1'//char(10)//cha
r(13)
call wribot(prmvlv(1:lcmmnd))
write(prmvlv,'(a,11,a)')'#1,SW',prm,'=0'//char(10)//cha
r(13)
waitasec='0:01.00'
call getstr(check,'Hit RETURN to close
valve','<CR>/',yescheck)
call wribot(prmvlv(1:lcmmnd))
call waiter(waitasec)
call wribot(vacprm(1:lcmmnd))
goto 89

continue

return

end !prime_reagents
*****
subroutine SOLENOIDS(colmax,down,ld,lu,NC,rowmax,up)
gives user direct control over individual solenoids

integer*4      colmax,rowmax,c,r,NC
logical        yescheck
integer*4      lu(colmax,rowmax), ld(colmax,rowmax)
character*60    up(colmax,rowmax), down(colmax,rowmax)
character*60    cmmnd

print*, ' '
print*, 'This routine allows you to control individual
solenoids.'
print*, 'All tips will automatically lift when you exit
the routine.'
print*, ' '

print*, 'Enter 999 to quit.'
call getlint(c,'Column:',999)
if (c.eq.999)goto 885
if (c.gt.colmax.or.c.lt.1) then
    print*, 'Input out of range.'
    goto 888
end if

call getlint(r,Row:,1)

```

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```
if (r.eq.999)goto 885
if (r.gt.rowmax,or.r.lt.1) then
    print*, 'Input out of range.'
    goto 887
end if

call getstr(cmmnd,'U or D:','U',yescheck)

if (cmmnd,eq.'999')goto 885
if (cmmnd,eq.'U').or.)(cmmnd.eq.'u')) then
    call wribot(up    r)(1:lu(c,r)))
    goto 888
end if !up
if ((cmmnd.eq.'D').or.(cmmnd.eq.'d')) then
    call wribot(down(c,r)(1:ld(c,r)))
    goto 888
end if !down
print*, 'Input out of range.'
goto 886

do c=1,NC
    do r=1,rowmax
        call wribot(up9c,r)(1:lu(c,r)))
    end do !r
end do !c

Continue

return

end !solenoids
*****
```

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What is claimed is:

1. An automated nucleic acid sequencer comprising:
  - a synthesizer for synthesizing oligomers;
  - means for hybridizing synthesized oligomers to sequencing patterns immobilized on membranes;
  - means for detecting hybridized sequencing patterns;
  - means for analyzing said detected patterns to construct a nucleic acid sequence; and
  - means for predicting next oligomers for subsequent synthesis, hybridization, detection and analysis.
2. The automated sequencer according to claim 1, wherein said synthesizer simultaneously synthesizes at least two oligomers having different sequences.
3. The automated sequencer according to claim 1, wherein said means for hybridizing simultaneously hybridizes at least two oligomers having different sequences to respective selected membranes containing immobilized sequencing patterns.
4. The automated sequencer according to claim 1, wherein said means for detecting simultaneously detects hybridized sequencing patterns on at least two membranes.
5. The automated sequencer according to claim 1, wherein an oligomer is hybridized to sequencing patterns bound to a first membrane at the

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same time that hybridized sequencing patterns on a second membrane are detected.

6. The automated sequencer according to claim 1, wherein a first oligomer is hybridized to sequencing patterns bound to a first membrane at the same time that a second oligomer is synthesized.

7. An automated nucleic acid sequencer comprising:

means for predicting oligomers to be synthesized;

a synthesizer for synthesizing at least two oligomers of predicted sequence simultaneously;

a plurality of membranes, each membrane containing immobilized sequencing patterns;

means for selecting at least one of said membranes for hybridization with each of said synthesized oligomers;

means for hybridizing said synthesized oligomers to the sequencing patterns immobilized on said selected membranes;

means for detecting hybridized sequencing patterns; and

means for analyzing said detected patterns to construct a nucleic acid sequence.

8. The automated sequencer according to claim 7, wherein said means for hybridizing simultaneously hybridizes at least two said oligomers to the sequencing patterns immobilized on respective selected membranes.

9. The automated sequencer according to claim 7, wherein said means for detecting

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simultaneously detects hybridized sequencing patterns on at least two membranes.

10. The automated sequencer according to claim 7, wherein an oligomer is hybridized to the sequencing patterns immobilized on a first membrane at the same time that the hybridized sequencing patterns immobilized on a second membrane are detected.

11. The automated sequencer according to claim 7, wherein a first oligomer is hybridized to sequencing patterns immobilized on a first membrane at the same time that a second oligomer is synthesized.

12. An automated nucleic acid sequencer comprising:

means for synthesizing a set of at least two oligomers of predicted sequence simultaneously;

means for labelling said oligomers;

an array containing a plurality of membranes, each said membrane containing immobilized sequencing patterns;

means for transporting each said labelled oligomer to a selected one of said plurality of membranes for hybridization to said immobilized sequencing patterns;

a detector for detecting hybridized sequencing patterns; and

a computer

for determining a portion of the sequence of said nucleic acid from said hybridized sequencing patterns;

for checking said sequence;

for predicting a next set of oligomers to be synthesized and hybridized to

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sequencing patterns to extend the determined sequence;

for selecting next membranes for hybridization with said predicted next set of oligomers; and

for directing the operation of said sequencer.

13. The automated sequencer according to claim 12, wherein at least two predicted oligomers are synthesized simultaneously by a single synthesizer.

14. A membrane unit which comprises:

a backing plate having a substantially planar front face and margins, said membrane being bonded to said face within said margins;

a flexible cover sheet bonded to said plate at said margins and extending over said face, enclosing said membrane between said face and said sheet; and

tubes attached to said sheet to form inlet and outlet ports for permitting fluids to flow between said sheet and said membrane.

15. The membrane unit according to claim 14, wherein a cover plate oriented in a plane substantially parallel to the plane of said face opposes said face and is moveable normal to the plane of said face to contact and to move said sheet towards said membrane to limit the separation between said membrane and said sheet when fluid is placed between said membrane and said sheet.

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16. The automated sequencer according to claim 12, comprising a membrane unit according to claim 14.

17. The automated sequencer according to claim 12, comprising a membrane unit according to claim 15, wherein a detection element is inserted between said sheet and said cover plate.

18. The automated sequencer according to claim 12, wherein said membrane units are adapted to extend from said array to be presented to a detector for detection of hybridized sequencing patterns.

19. An automated sequencer for nucleic acid sequencing using a multiplex walking technique comprising:

an oligomer synthesizer for synthesizing and labelling oligomers of at least two sequences simultaneously;

a membrane unit array comprising a plurality of membrane units, each membrane unit comprising a membrane containing immobilized multiplex sequencing patterns, said membrane units being adapted for multiple independent hybridizations with different oligomers;

means for transporting each labelled oligomer from said synthesizer to a selected membrane unit for hybridization;

a detector for detecting hybridized sequencing patterns on at least two membranes simultaneously; and

a computer

for predicting a set of oligomers;

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for directing said synthesizer to synthesize and label said oligomers;

for selecting membrane units for hybridization with each said predicted oligomer;

for directing transport of said oligomers to said selected membrane units;

for directing hybridization of said oligomers to multiplex sequencing patterns immobilized on membranes contained within said membrane units;

for analyzing said hybridized multiplex sequencing patterns to determine short nucleic acid sequences, combining said sequences to construct a longer nucleic acid sequence, and checking said longer nucleic acid sequence for error; and

for predicting a next set of oligomers, and selecting next membrane units for hybridization with said next set of oligomers;

wherein one of said oligomers is hybridized in a first selected membrane unit simultaneously with synthesis of one of said next oligomers for hybridization within a second selected membrane unit and presentation of a third hybridized membrane unit to said detector.

20. An automated method for nucleic acid sequencing, which comprises the steps of:

synthesizing oligomers;

hybridizing synthesized oligomers to sequencing patterns immobilized on membranes;

detecting hybridized sequencing patterns;

analyzing said detected patterns to construct a nucleic acid sequence; and

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predicting the sequence of next oligomers for subsequent synthesis, hybridization, detection and analysis.

21. The method according to claim 20, wherein oligomers having different sequences are synthesized simultaneously.

22. The method according to claim 20, wherein at least two oligomers having different sequences are hybridized simultaneously to respective selected membranes containing immobilized sequencing patterns.

23. The method according to claim 20, wherein at least two membranes are simultaneously presented for detection of hybridized sequencing patterns.

24. The method according to claim 20, wherein at least two steps occur simultaneously on respective of said membranes.

25. The method according to claim 24, wherein an oligomer is hybridized to sequencing patterns immobilized on a first membrane at the same time that the hybridized sequencing patterns on a second membrane are detected.

26. The method according to claim 24, wherein a first oligomer is hybridized to sequencing patterns immobilized on a first membrane at the same time that a second oligomer is synthesized.

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27. An automated method for nucleic acid sequencing using a plurality of membranes, each membrane containing immobilized sequencing patterns, which comprises the steps of:

predicting oligomers to be synthesized;  
synthesizing at least two oligomers simultaneously;

selecting at least one of said membranes to correspond to each of said synthesized oligomers;

hybridizing said synthesized oligomers to the sequencing patterns immobilized on said selected membranes;

detecting hybridized sequencing patterns;

analyzing said detected patterns to construct a nucleic acid sequence.

28. The method according to claim 27, wherein at least two said synthesized oligomers are simultaneously hybridized to the sequencing patterns immobilized on at least two respective selected membranes.

29. The method according to claim 27, wherein the hybridized sequencing patterns on at least two membranes are simultaneously detected.

30. The method according to claim 27, wherein at least two steps occur simultaneously on respective of said plurality of membranes.

31. The method according to claim 30, wherein an oligomer is hybridized to the sequencing patterns immobilized on a first membrane at the same

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time that the hybridized sequencing patterns on a second membrane are detected.

32. The method according to claim 30, wherein a first oligomer is hybridized to sequencing patterns immobilized on a first membrane at the same time that a second oligomer is synthesized.

33. An automated method for nucleic acid sequencing with a multiplex walking technique using a plurality of membrane units, each containing a membrane upon which multiplex sequencing patterns have been immobilized, which comprises the steps of:

predicting a set of oligomers for synthesis and hybridization;

selecting membrane units for hybridization with said predicted oligomers, each said selected membrane unit corresponding to one of said predicted oligomers;

synthesizing oligomers of at least two different sequences simultaneously;

eluting, purifying and labelling said synthesized oligomers;

transporting each labelled oligomer to a selected membrane unit for hybridization;

hybridizing said transported oligomers to sequencing patterns immobilized on membranes contained within respective selected membrane units;

detecting the hybridized sequencing patterns on at least two membranes simultaneously;

analyzing each said detected pattern to determine a short nucleic acid sequence;

combining said short nucleic acid sequences to construct a longer nucleic acid sequence;

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checking said longer nucleic acid sequence for error; and

predicting a next set of oligomers and selecting a next set of membrane units for hybridization with said next set of oligomers, wherein one of said predicted oligomers is hybridized in a first specified membrane unit simultaneously with synthesis of one of said next predicted oligomers for hybridization within a second specified membrane unit and with detection of hybridized sequencing patterns in a third membrane unit.

34. The use of nucleic acid sequence information generated by a method according to any one of claims 19-32, to prepare a nucleic acid molecule having at least a portion of its sequence based on said nucleic acid sequence data.

35. The use of a nucleic acid molecule having at least a portion of its sequence based on nucleic acid sequence information generated by a method according to any one of claims 19-32.

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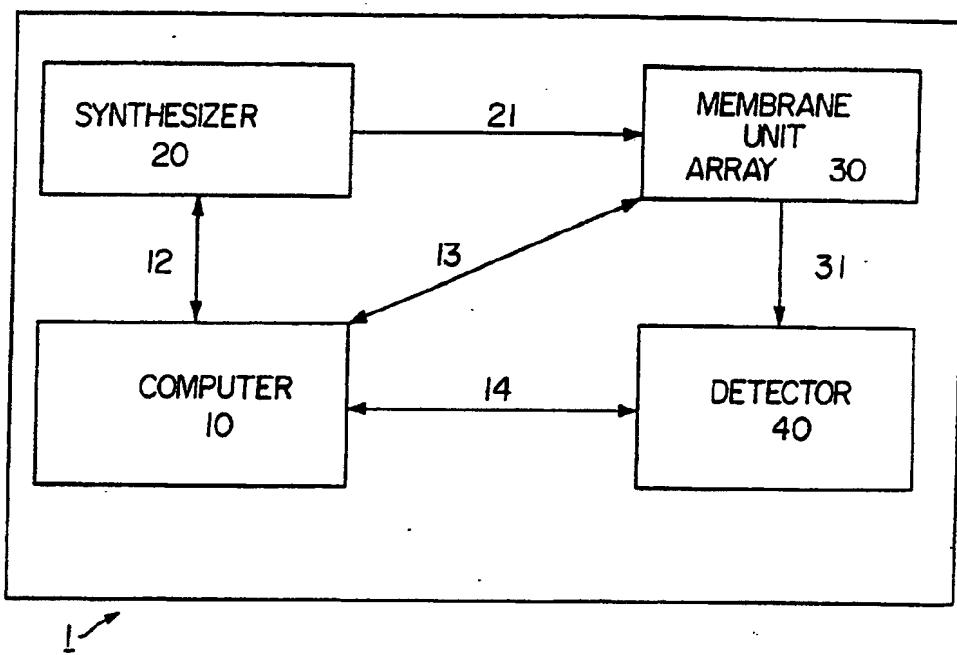


FIG. 1

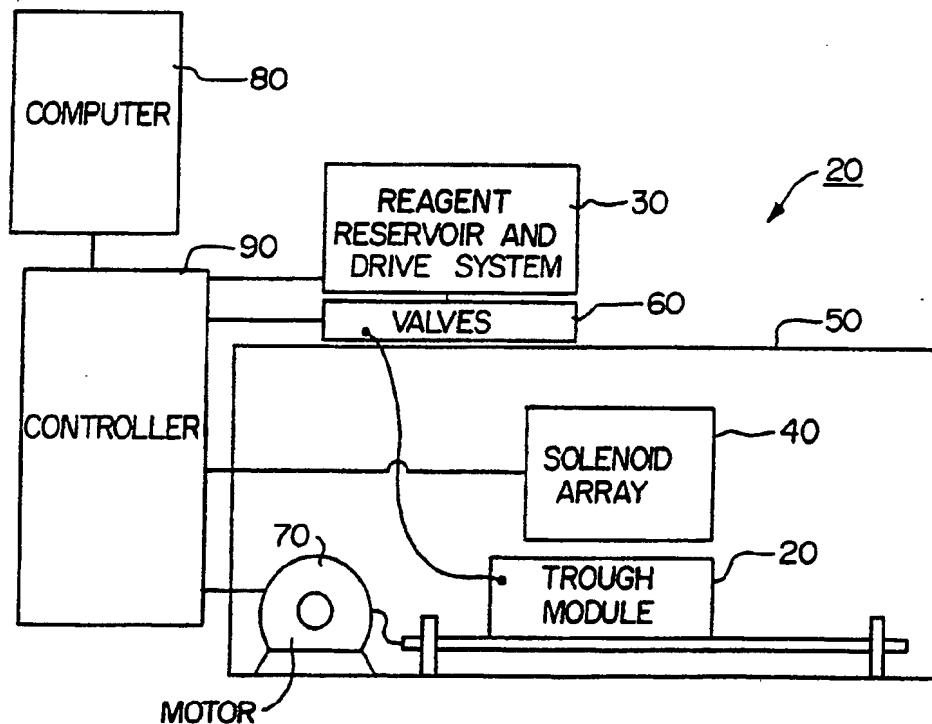


FIG. 2

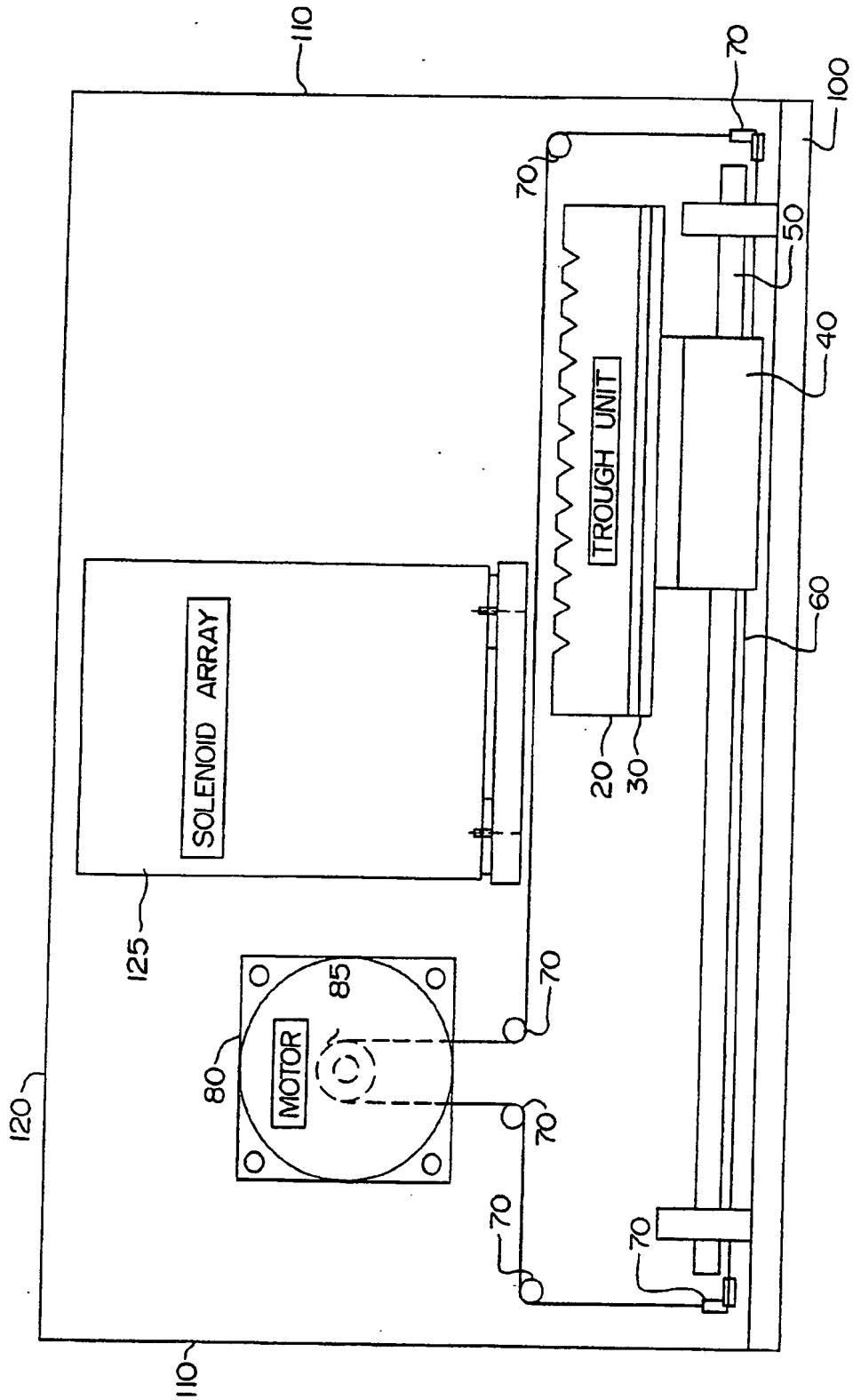


FIG. 3

FIG. 4B

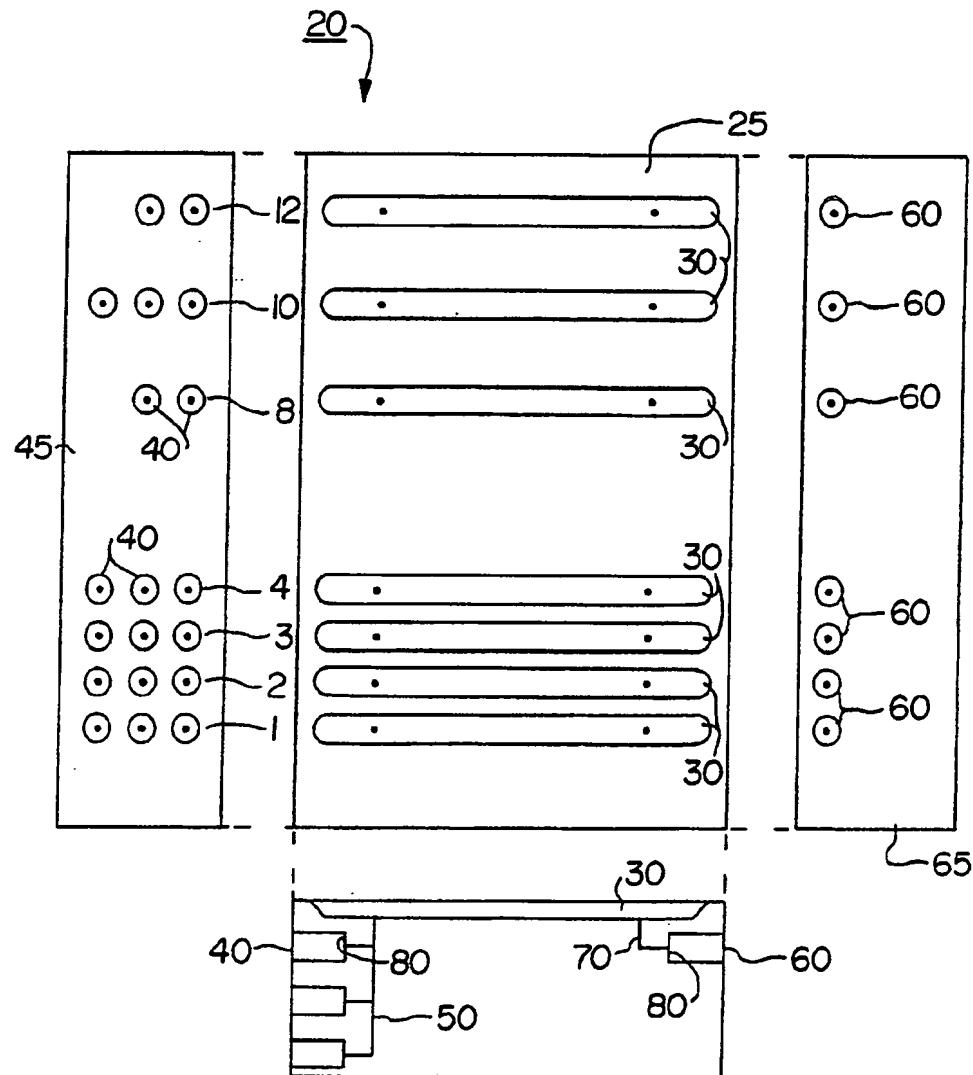


FIG. 4A

FIG. 4C

FIG. 4D

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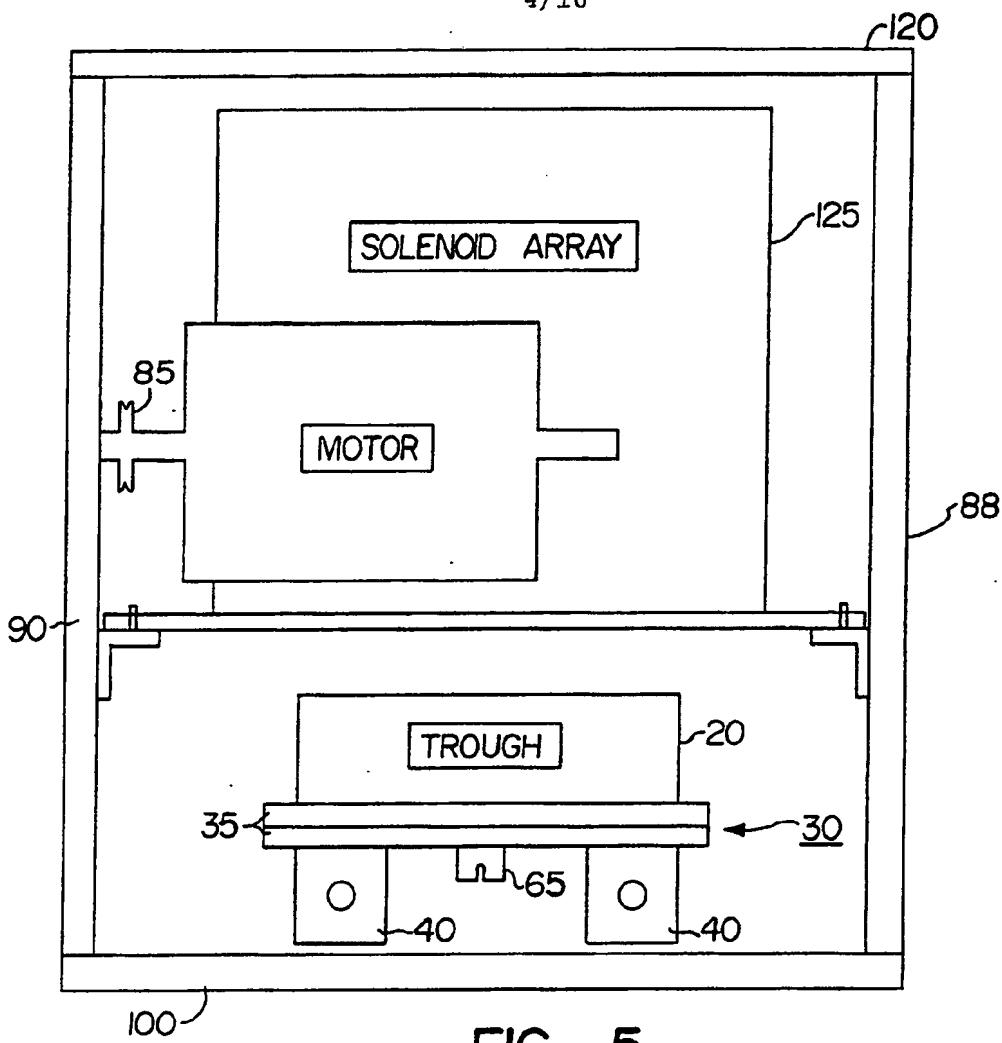


FIG. 5

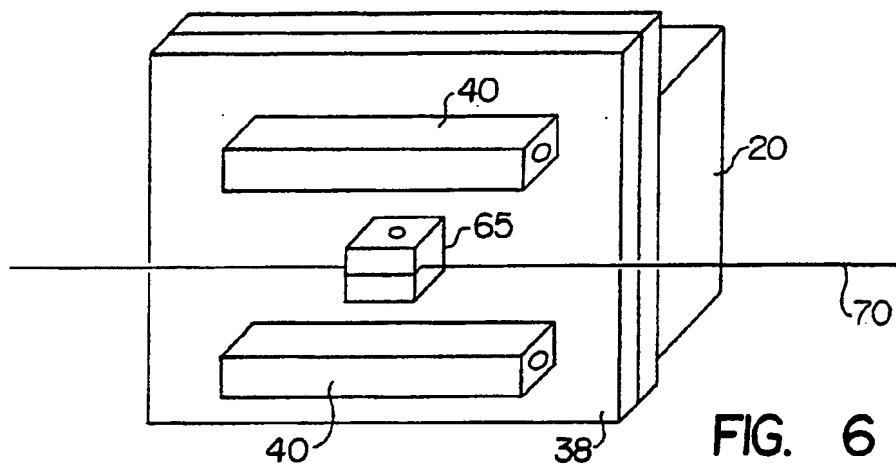


FIG. 6

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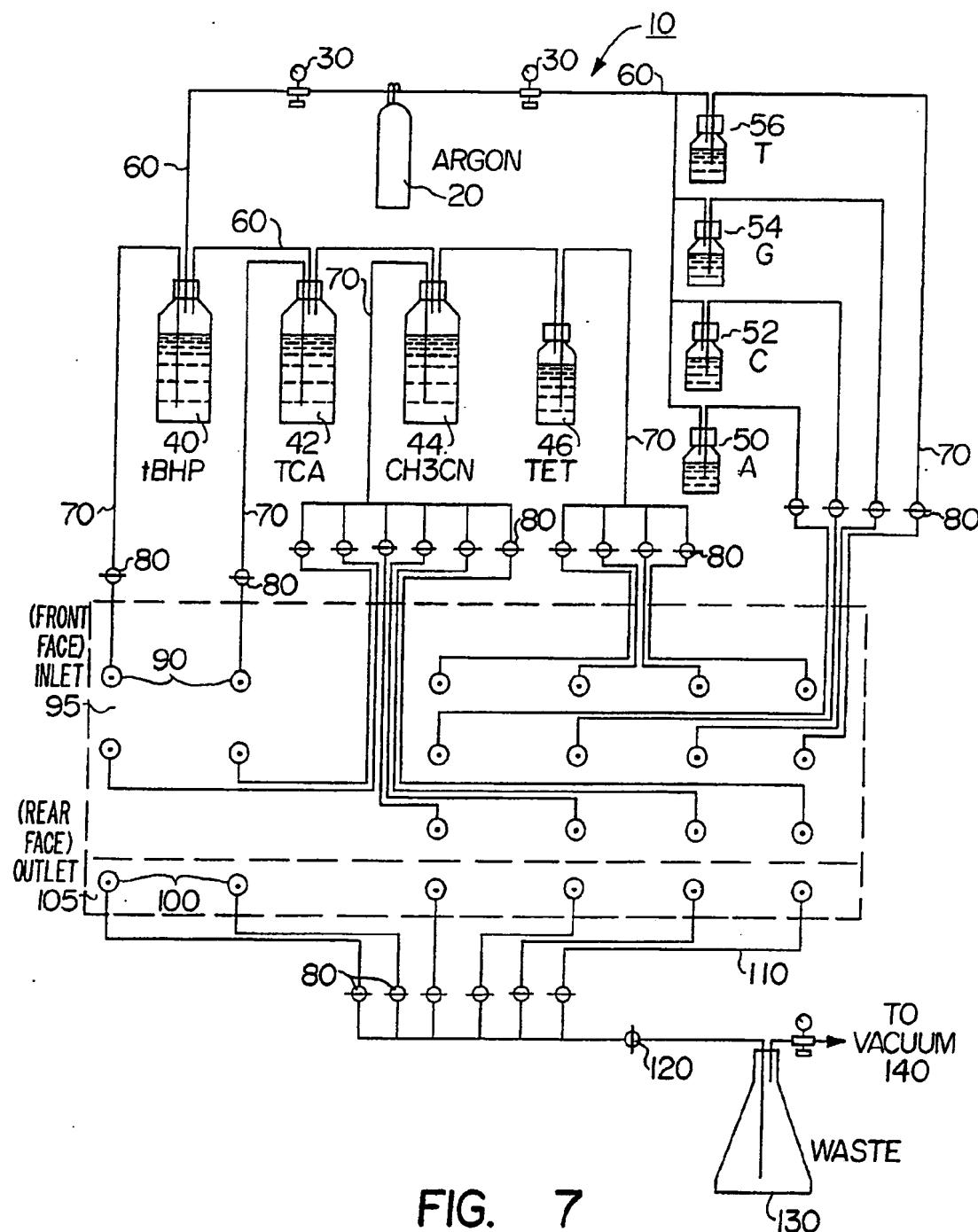


FIG. 7

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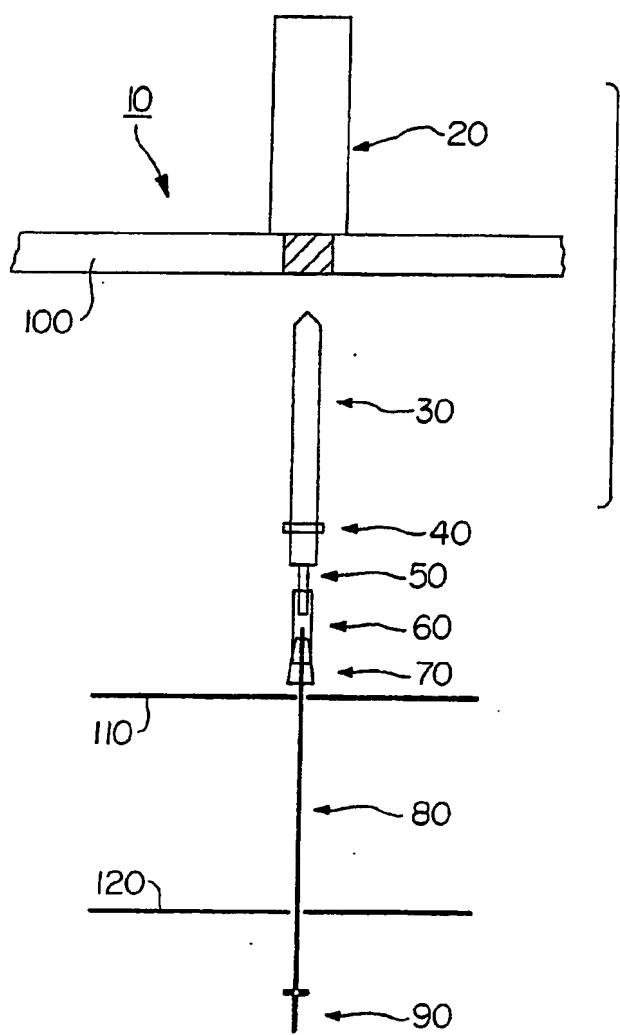


FIG. 8

**SUBSTITUTE SHEET****CGK00001979**

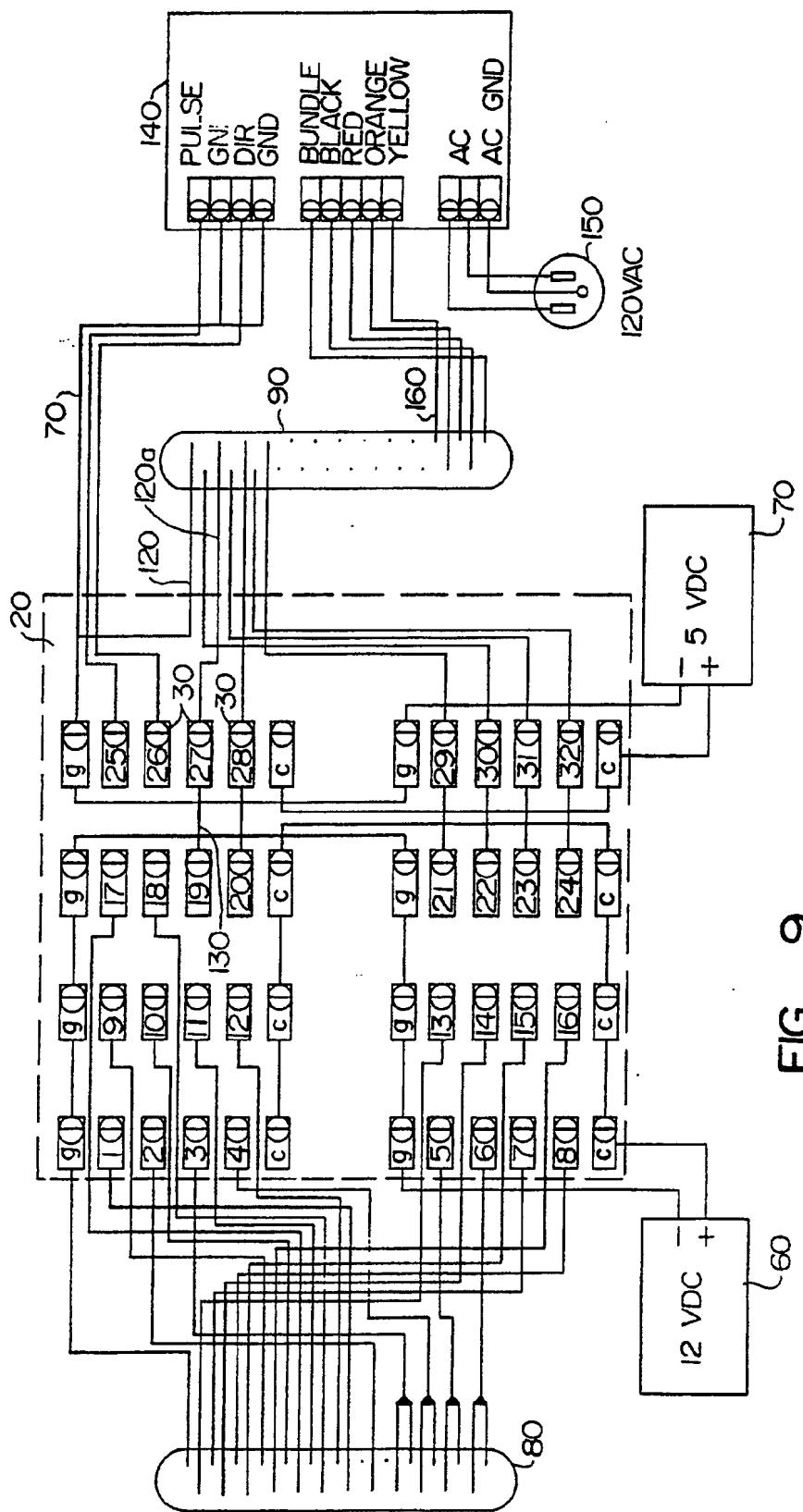


FIG. 9

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CGK00001980

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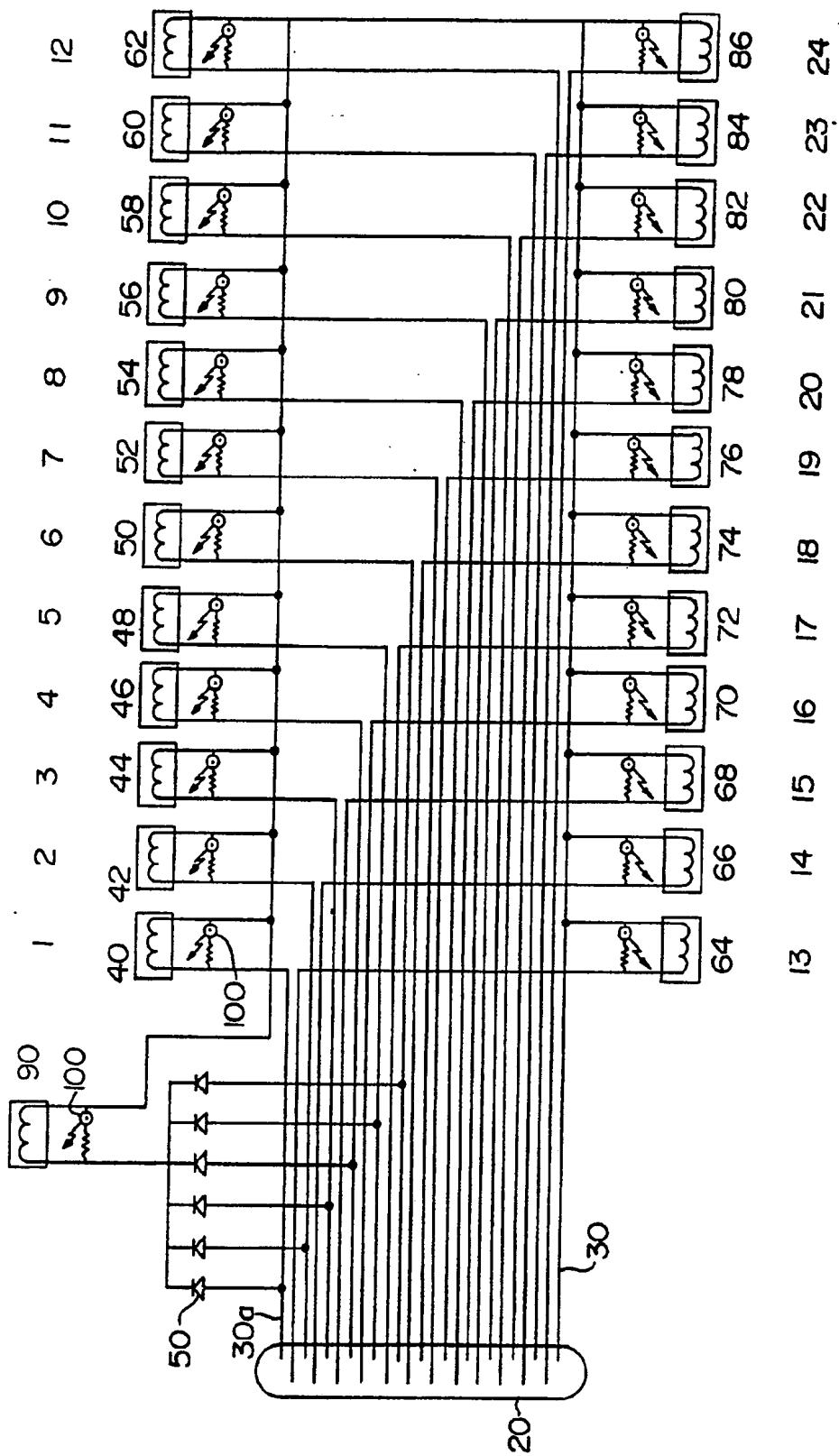


FIG. 10

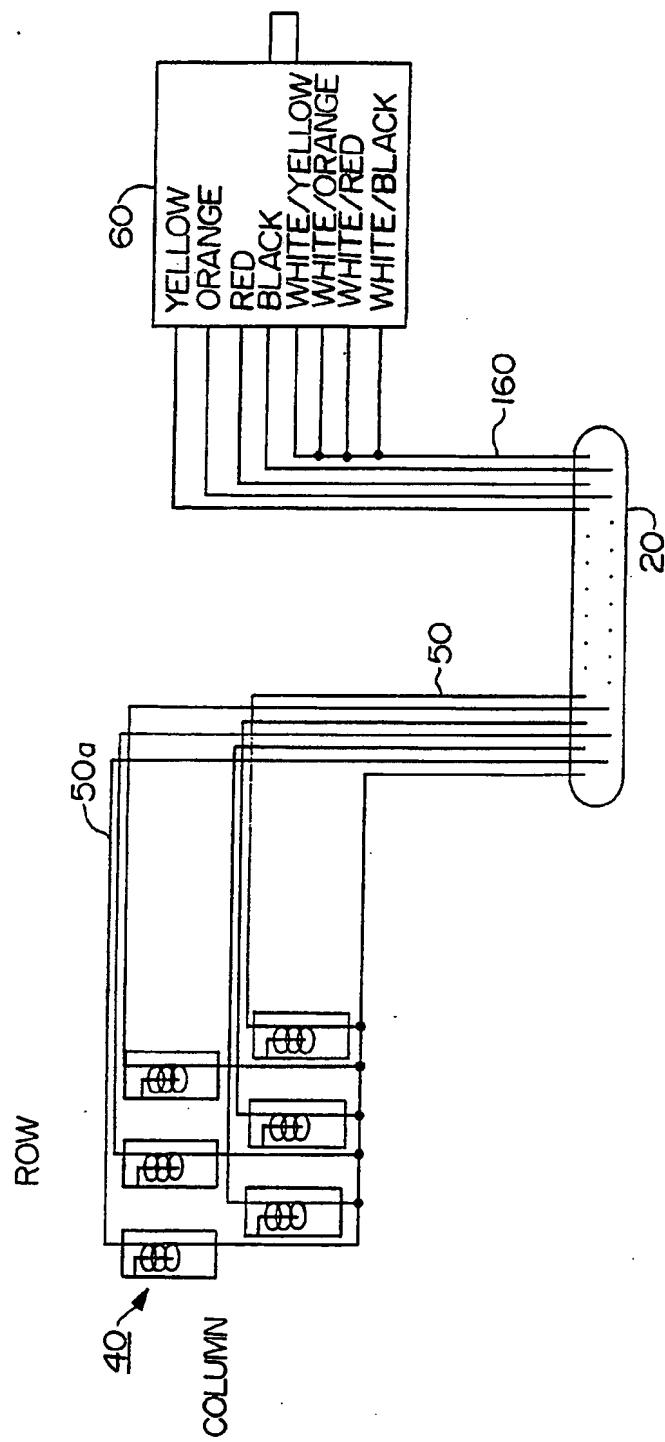


FIG. 11

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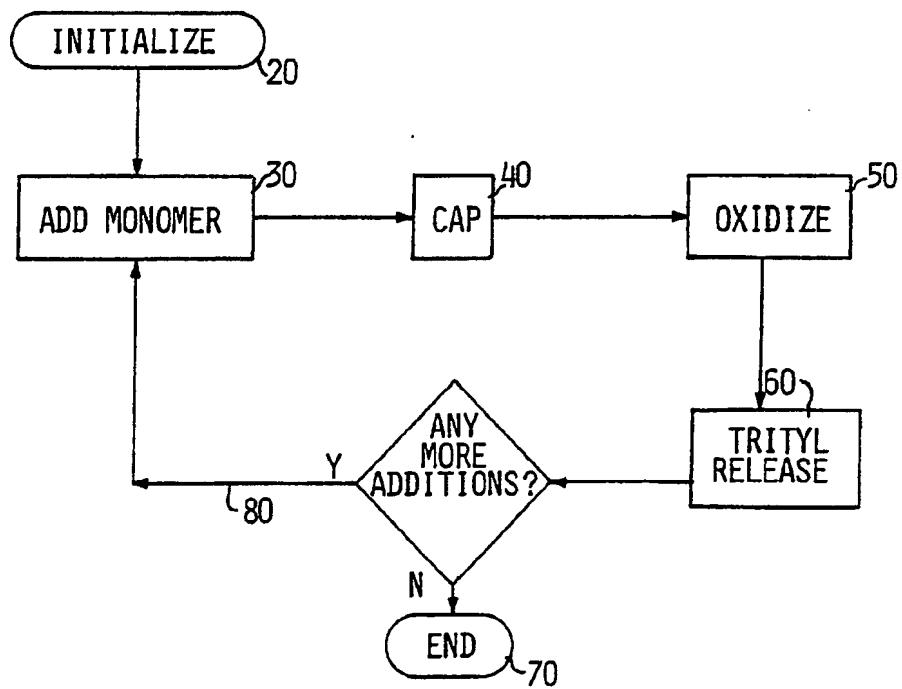


FIG. 12A

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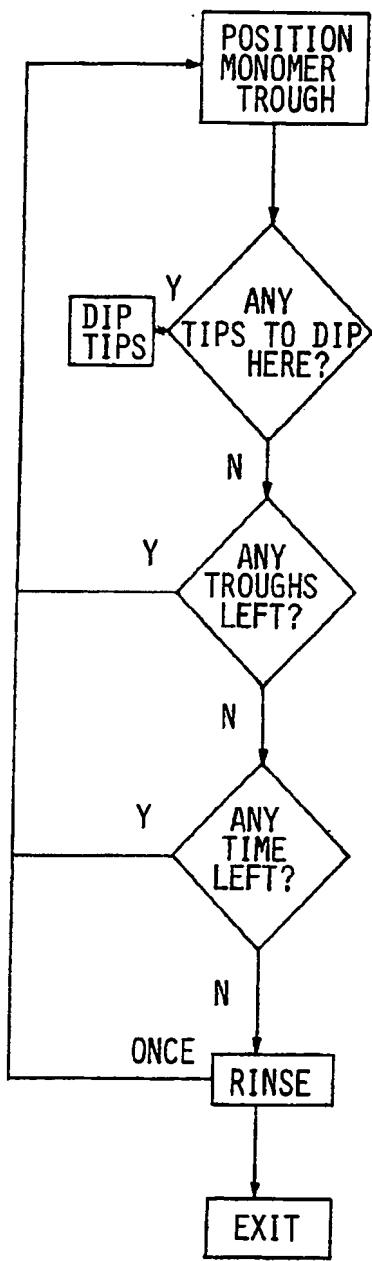


FIG. I2B

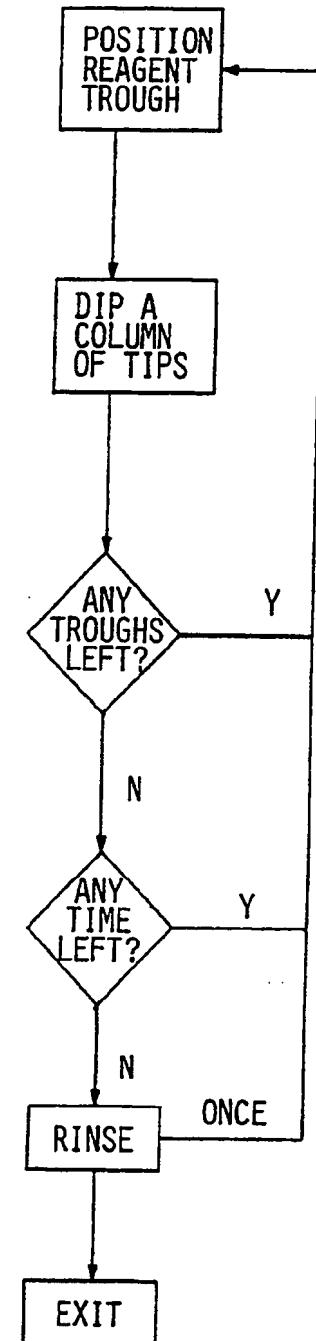
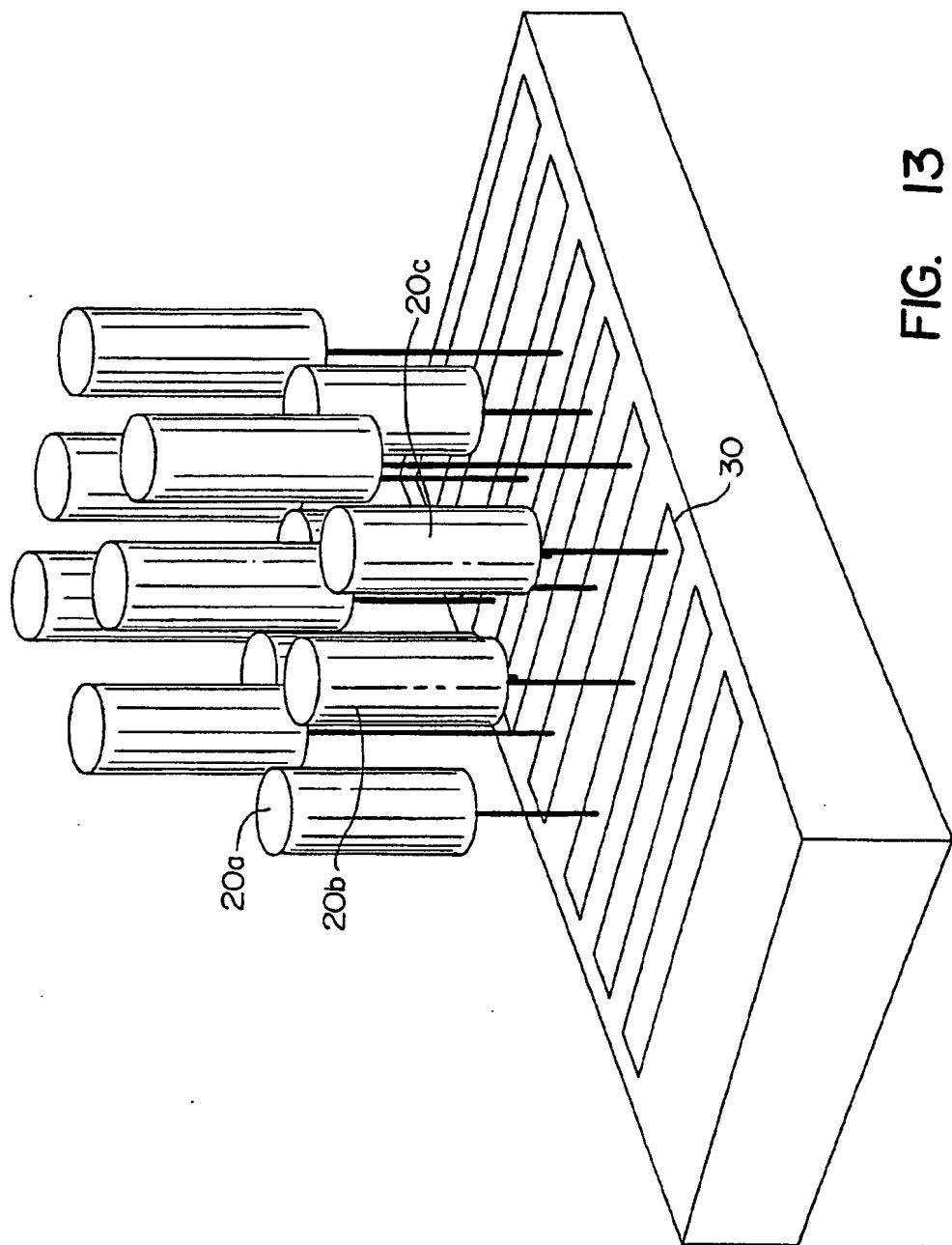


FIG. I2C

FIG. 13



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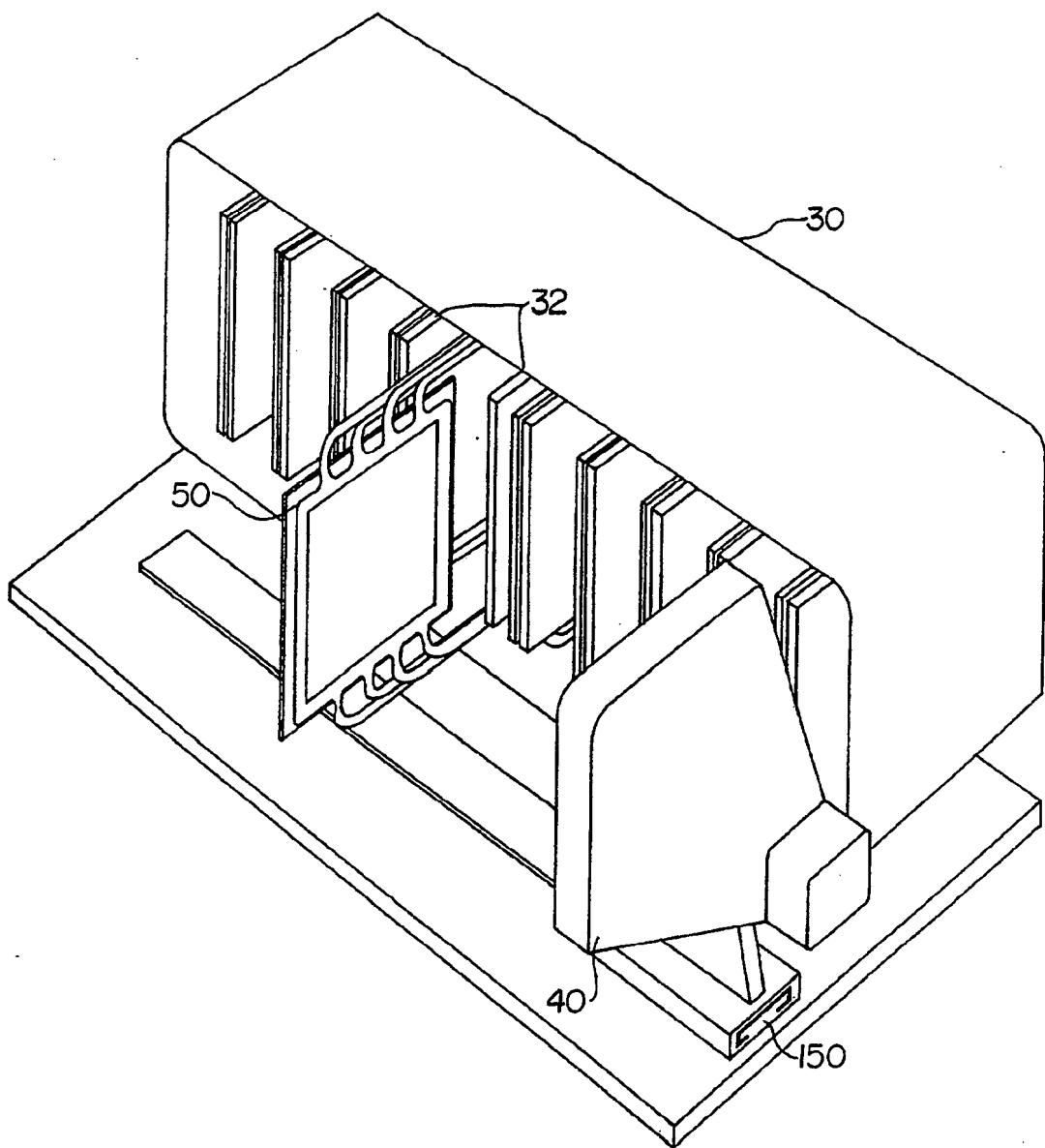


FIG. 14

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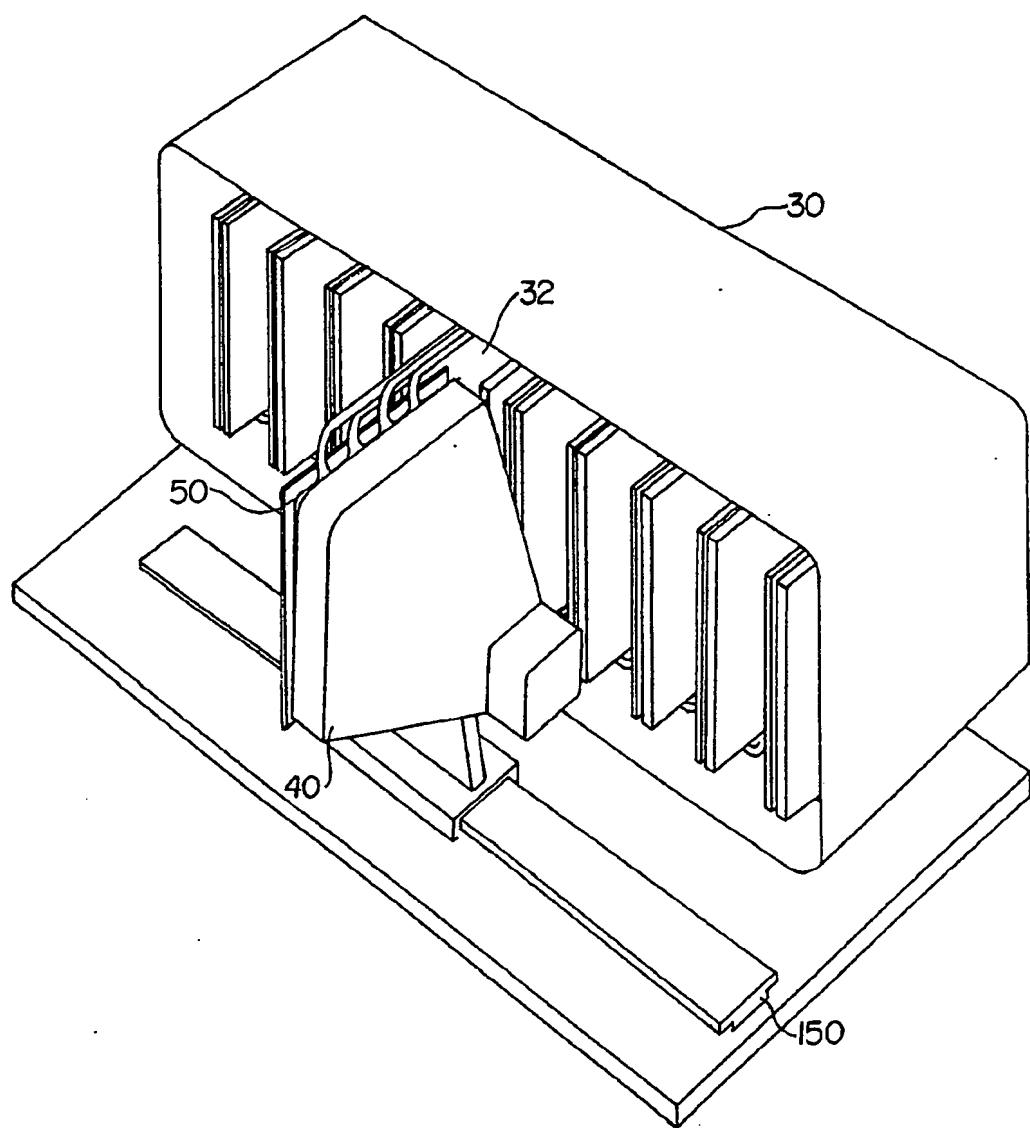


FIG. 15

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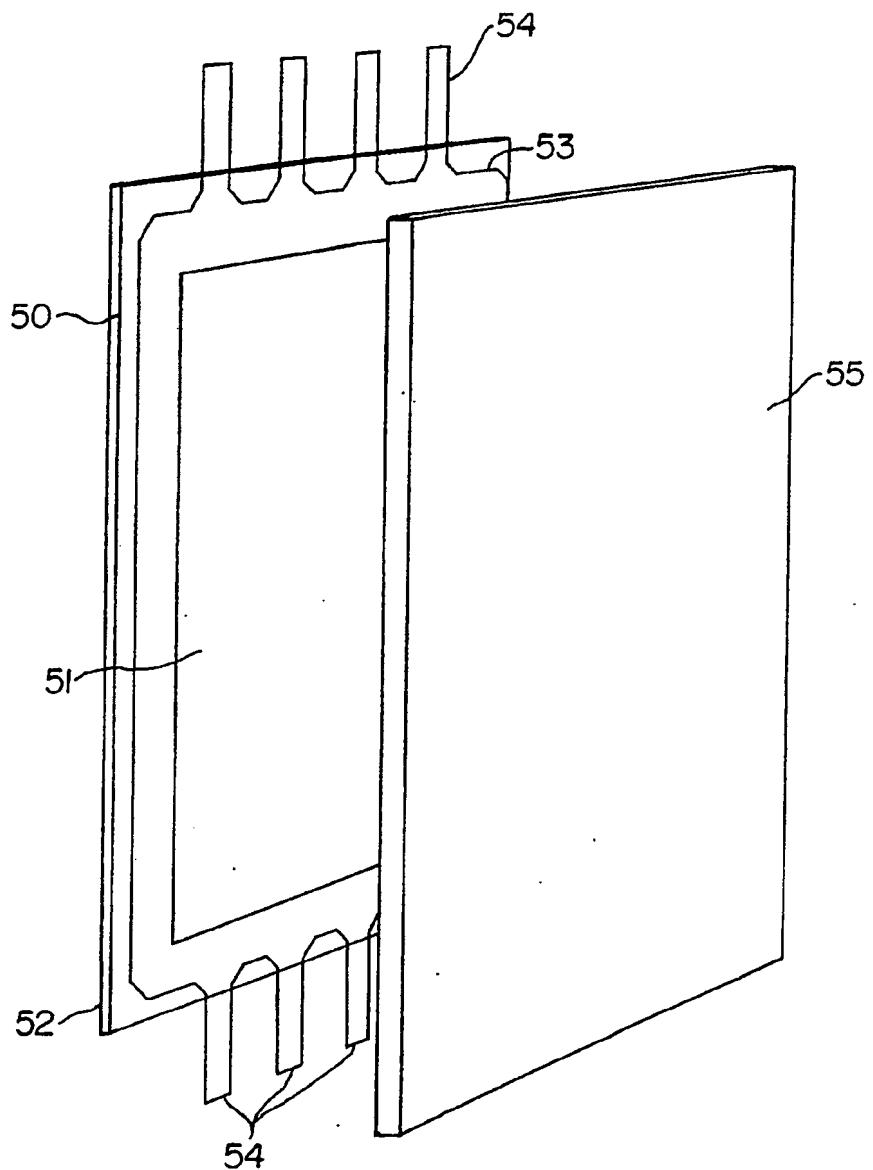


FIG. 16

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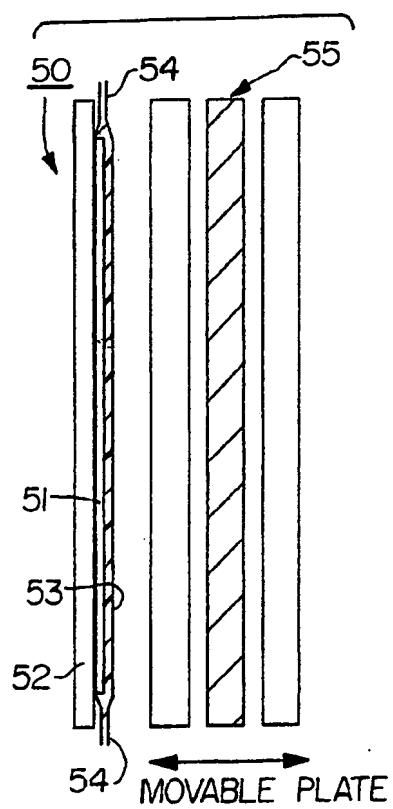


FIG. 17A

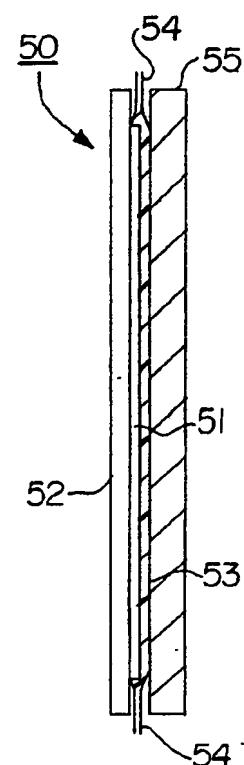


FIG. 17B

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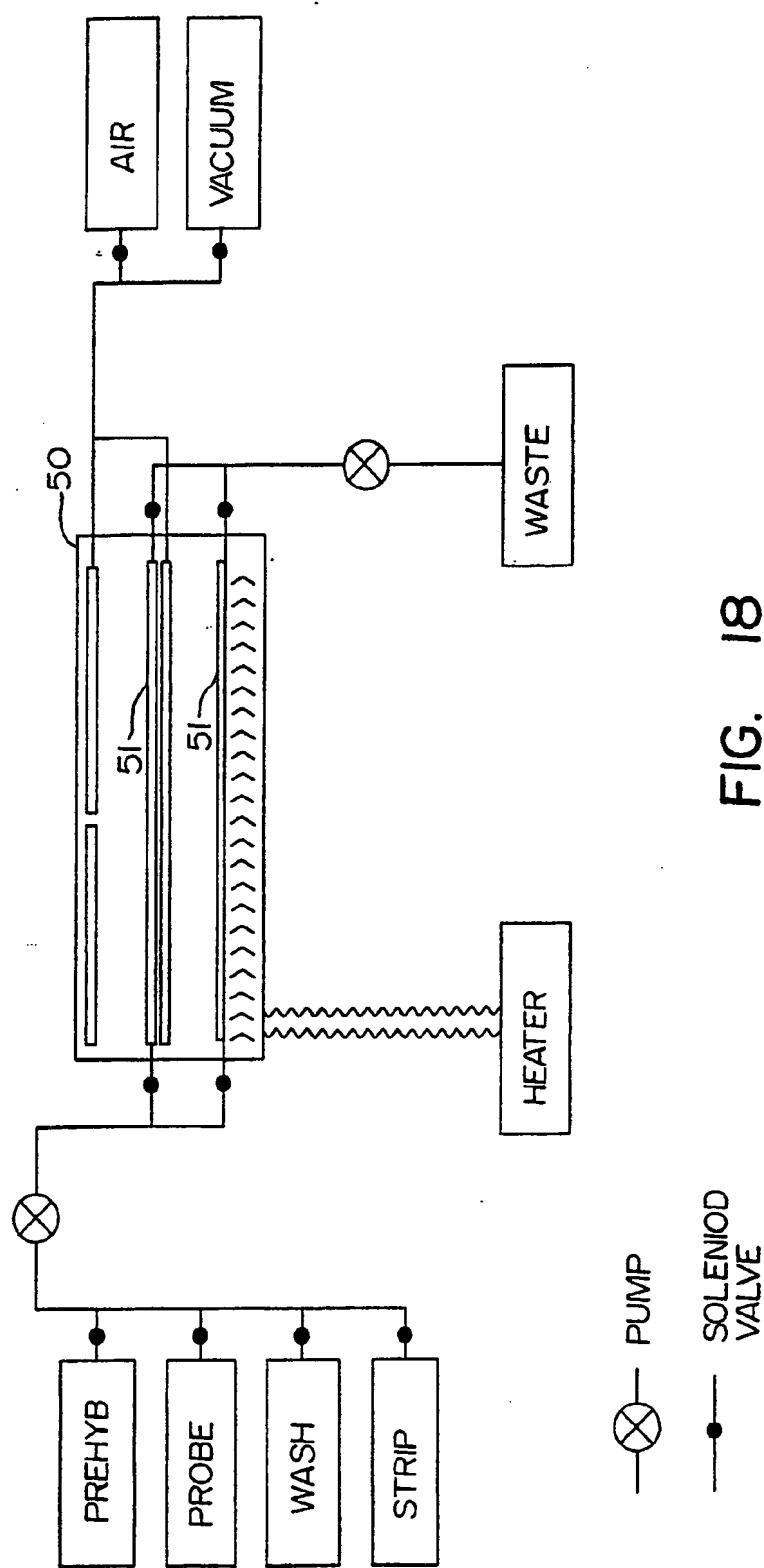


FIG. 18

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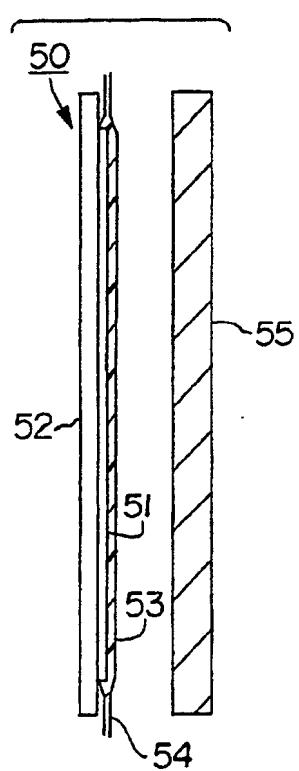


FIG. 19A

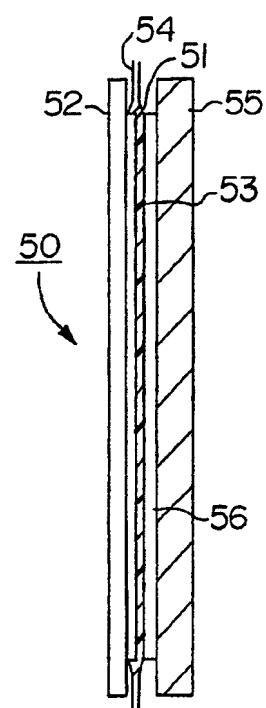


FIG. 19B

**SUBSTITUTE SHEET****CGK00001991**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1. 5 C12Q1/68; //G01N35/00

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols
Int.C1. 5	C12Q ; G01N

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EP,A,0 303 459 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 15 February 1989 see column 6, line 59 - column 12, line 44; figure 7	14,15
Y A	& US,A,4 942 124 17 July 1990 cited in the application ---	1 2-12, 16-18
Y A	WO,A,9 106 678 (SRI INTERNATIONAL) 16 May 1991 see page 11, line 28 - page 18, line 23; claim 50; figure 4 ---	1 7,12
		---

<sup>10</sup> Special categories of cited documents :<sup>10</sup><sup>"A"</sup> document defining the general state of the art which is not considered to be of particular relevance<sup>"E"</sup> earlier document but published on or after the international filing date<sup>"L"</sup> document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<sup>"O"</sup> document referring to an oral disclosure, use, exhibition or other means<sup>"P"</sup> document published prior to the international filing date but later than the priority date claimed<sup>"T"</sup> later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<sup>"X"</sup> document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step<sup>"Y"</sup> document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.<sup>"Z"</sup> document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

04 SEPTEMBER 1992

Date of Mailing of this International Search Report

06 OCT 1992

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

DE KOK A.J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, September 1989, WASHINGTON US pages 6883 - 6887; O.OHARA ET AL.: 'Direct genomic sequencing of bacterial DNA: The pyruvate kinase I gene of <i>Escherichia coli</i> ' cited in the application see the whole document	20
A	---	1,7,12, 19
Y	BIOTECHNOLOGY vol. 8, December 1990, NEW YORK US pages 1251 - 1256; W.BAINS: 'Alternative routes through the genome' see page 1252, column 1, paragraph 3 - page 1256, paragraph 2; figures 3,6	20
A	---	1
X	DE,A,3 635 966 (HOEFER SCIENTIFIC INSTRUMENTS) 21 May 1987 see the whole document	14,15
A	DE,A,3 306 770 (SHIMADZU CORP.) 15 September 1983 see the whole document	1
A	DE,A,3 805 808 (EUROPÄISCHES LABORATORIUM FÜR MOLEKULARBIOLOGIE (EMBL)) 7 September 1989 see the whole document	1,12
T	SCIENCE. vol. 254, no. 5028, 4 October 1991, LANCASTER, PA US pages 59 - 67; T.HUNKAPILLER ET AL.: 'Large-scale and automated DNA sequence determination' see the whole document	1-31

**ANNEX TO THE INTERNATIONAL SEARCH REPORT**  
**ON INTERNATIONAL PATENT APPLICATION NO. US 9204339**  
**SA 60713**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 04/09/92

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0303459	15-02-89	US-A- 4942124 JP-A- 1137982	17-07-90 30-05-89	
US-A-4942124	17-07-90	EP-A- 0303459 JP-A- 1137982	15-02-89 30-05-89	
WO-A-9106678	16-05-91	EP-A- 0450060		09-10-91
DE-A-3635966	21-05-87	US-A- 4681853 JP-A- 62116261	21-07-87 27-05-87	
DE-A-3306770	15-09-83	JP-C- 1464554 JP-A- 58148896 JP-B- 63011360 JP-C- 1432128 JP-A- 58148897 JP-B- 62041676 JP-C- 1353471 JP-A- 58148898 JP-B- 61021639 JP-C- 1434321 JP-A- 58157799 JP-B- 62041677 CA-A- 1199776 GB-A, B 2118189	28-10-88 05-09-83 14-03-88 24-03-88 05-09-83 03-09-87 11-12-86 05-09-83 28-05-86 07-04-88 19-09-83 03-09-87 28-01-86 26-10-83	
DE-A-3805808	07-09-89	None		